Gene expression of xenobiotic metabolising enzymes in rat liver and kidney: Differential effects of rooibos and honeybush herbal teas

S Abrahams

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Department of Medical Biosciences
Faculty of Natural Sciences
University of the Western Cape

Study Leader: Prof. WCA Gelderblom
Co-study Leaders: Prof. E Joubert
Prof. M de Kock

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DECLARATION

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

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S Abrahams Date

ABSTRACT

Laboratory studies, epidemiological investigations and human clinical trials indicate that flavonoids have important effects on cancer chemoprevention and therapy. Flavonoids may interfere in several steps that lead to cancer development but may also lead to toxicity as the inhibition of carcinogen-activating enzymes may also cause potential toxic flavonoid-drug interactions. However, the potential toxicity of these dietary components has not been well studied. The use of polyphenol-enriched supplements prepared from South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) are being marketed to alleviate symptoms that are known to be "cured" by the herbal teas. However, there is a lack of information regarding the possible health promoting effects of these polyphenol-enriched extracts on xenobiotic metabolism. In the present study, the modulating effects of aspalathin-enriched rooibos and mangiferin-enriched C. *genistoides* and *C. subternata* extracts on the gene expression of xenobiotic metabolising enzymes (XMEs) were investigated *in vivo* in the rat liver and kidneys. An *in vitro* study, utilising a primary rat hepatocyte cell model, was included to further evaluate changes in the expression of selected XMEs by the herbal tea extracts, including their major polyphenolic constituents, aspalathin and mangiferin. The use of the *in vitro* primary hepatocytes assay as a predictive cell model for the modulation of the expression of XMEs genes by the herbal tea extracts *in vivo* was critically evaluated.

In the liver and kidneys, the *C. subternata* polyphenol-enriched herbal tea extract effected the majority of changes regarding the expression of XMEs genes when compared to the rooibos and *C. genistoides*. Variations in the modulation of gene expression of the XMEs by the herbal tea extracts were related to differences in their polyphenol constituents, although non-polyphenolic constituent could also be involved. Overall the herbal teas regulated alcohol, energy, drug and steroid metabolism in the liver, whereas in the kidneys the gene expression of phase I, phase II, steroid metabolising enzymes, as well as drug transporters were modulated. It would appear that the herbal teas are likely to exhibit both beneficial and adverse effects *in vivo*,

depending on the specific organ, the xenobiotic and/or drug that are involved. The primary rat hepatocytes model display varying effects with respect to modulating gene expression of specific XMEs by the polyphenol-enriched herbal tea extracts. Apart from the reduction in 17β-hydroxysteroid dehydrogenase gene expression care should be taken to directly extrapolate the *in vitro* findings to changes that prevail *in vivo*. However, interesting results regarding the masking effect of complex mixture on the modulation of XME gene expression of individual polyphenols were encountered. In addition differences in the dose and duration of exposure between the *in vitro* and *in vivo* studies were not comparable and should be further explored to validate the *in vitro* primary hepatocytes model to predict changes *in vivo*. Future studies should investigate the effects of the herbal tea extracts, its polyphenols and metabolites on XME induction at a protein level as well as varying herb-drug-enzyme interactions.

I wish to dedicate this thesis to my friends and family for their love and support

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AhR	Aryl hydrocarbon receptor
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APS	Adenosine S'- phosphosulphate
ARC	Agricultural Research Council
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
B[a]P	Benzo α pyrene
B-ME	Beta mercaptoethanol
CAM	Complementary and alternative medicine
CCl_4	Carbon tetrachloride
COOH	Carboxyl group
CYP	Cytochrome p450
COMT	Catechol-*O*-methyltransferase
C_{gen}	*C. genistoides*
C_{sub}	*C. subternata*
DMBA	7, 12-dimethylbenz [a] anthracene
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E_1	Estrone
E_2	Estradiol
ECRA	Ethics committee for research on animals
EGTA	Ethylene glycol tetra acetic acid

EMEM	Eagle's minimal essential medium
FBS	Fetal bovine serum
F-C	Folin-Ciocalteau
GA	Gallic acid
GFJ	Grapefruit juice
GIT	Gastrointestinal tract
GSH	Glutathione
GSSG	Reduced glutathione
GST	Glutathione S transferase
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine guanine phosphoribosyltrasferase
HSD	Hydroxysteroid dehydrogenase
ICR	Imprinting control region
IC_{50}	Inhibitory concentration (50%)
MRC	Medical Research Council
mRNA	Messenger ribonucleic acid
NAB	N'-Nitrosoanabasine
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
$NaHCO_3$	Sodium bicarbonate
Na_2CO_3	Disodium bicarbonate
N.A	Not applicable
NAT	N'-nitrosanatabine
NAT	N-acetyltransferase
NH_2	Amine group
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NNN	N'-Nitrosonornicotine
O_2	Molecular oxygen
OH	Hydroxyl group
PAH	Poly aromatic hydrocarbon
PAPS	3'- phosphoadenosine-5'-phosphosulphate
PB	Phenobarbital
PBS	Phosphate buffered saline
P-gp	P-glycoprotein
P-value	Probability value
PROMEC	Programme for mycotoxin and experimental carcinogenesis
PXR	Pregnane X receptor
PXRE	Xenobiotic response element
QPCR	Quantitative real-time PCR
RIN	RNA integrity number
Rb	Rooibos
RNA	Ribonucleic acid
RXR	9-cis retinoic acid receptor
SAM	s-adenosylmethione
SH	Thiol/sulfhydral group
SJW	St John's wort
SN-38	Oxidised irinotecan
SO_4	Sulphate group
SULT	Sulfotransferase
TPP	Total polyphenol
UDP	Uridyl diphosphate
UDPGT	UDP-glucuronyl transferase
UGT	UDP-glucuronyl transferase

WE Williams' Medium E

XMEs Xenobiotic metabolising enzymes*

*XME Gene abbreviations summarised in Addendum II

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Notes:

This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has therefore been unavoidable.

Abbreviations of the xenobiotic metabolising enzymes are capitalised, whereas gene abbreviations are in italics.

LIST OF ADDENDA

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another (Fugh-Berman, 2000).

For centuries, people have been using herbs as medicine for longevity and essential well being. Decoctions and mixtures are used to treat many illnesses such as respiratory and cardiac problems (Bocek, 1984). The World Health Organisation (WHO) estimates that 4 billion people, 80 percent of the world population, presently use herbal medicine for some aspect of primary health care (Farnsworth et al., 1985). Many patients use herbal products in combination with prescribed drugs, however, concurrent use of herbs may mimic, magnify, or oppose the effect of drugs (Fugh-Berman, 2000).

Pharmaceutical drugs and herbal preparations, amongst others, are considered foreign to the body, where it elicits various responses. These xenobiotics are metabolised by specific enzymes known as xenobiotic metabolising enzymes (XMEs), including cytochrome P450, which are present predominantly in the liver, but are also found in extrahepatic tissues such as the kidneys. Some xenobiotics are substrates to the same enzymes and thus compete for the same binding sites. Therefore, one xenobiotic may inhibit or enhance the metabolism of the other due to overlapping substrate specificity (Nebert, 1991). However, some metabolites or so-called reactive intermediates are known to be toxic, interact with cellular constituents such as the

DNA resulted in genotoxic effects and eventually carcinogenesis (reviewed in Nebert, 1991).

The most common means of regulating XMEs is transcriptional (Porter and Coon, 1991). The genes that code for XMEs are regulated by nuclear hormone receptors (Savas et al., 1999) which are turned on when there are elevated intracellular levels of exogenous ligands. An explanation for this is that XMEs could be responsible for controlling the steady state levels of xenobiotics (Nebert, 1991). However, xenobiotic metabolism is not the primary function of the XMEs as it also metabolises endogenous substrates such as steroids, due to the structural similarity between foreign chemicals and endogenous ligands. It is therefore important to study the effects on XMEs when exploring the health benefits of herbal preparations and/or its constituents. South African herbal teas, rooibos (*Aspalathus linearis)* and honeybush *(Cyclopia* spp.) are popular beverages worldwide (Snyman, 2000; De Villiers, 2004).

The increase in the herbal tea market is due to the known beneficial health properties of the teas, which is mainly anecdotal. Although scientific evidence is minimal with respect to the health benefits and effects of the herbal teas in humans, animal and *in vitro* studies have shown anti-oxidant, anti-mutagenic, anti-inflammatory and anti-carcinogenic properties (Marnewick *et al*., 2000; Marnewick *et al*., 2004; Joubert *et al*., 2008; Sissing *et al*., 2010). It is believed that these properties are attributed to the rich polyphenolic content of the herbal teas, with special reference to the major constituents, aspalathin in rooibos and mangiferin in honeybush tea. Polyphenol-enriched extracts have thus been introduced as supplements in an attempt to magnify the health beneficial effects observed after consumption of the herbal teas. However,

since polyphenols are known to interact with XMEs (Pirmohamed, 2008), it is imperative to investigate the effects of the polyphenol-enriched extracts on XME regulation, in order to deem it safe for consumption. The present thesis discusses the effects of aspalathin-enriched rooibos and mangiferin-enriched extracts of two species of *Cyclopia*, *C. genistoides* and *C. subternata*, on the gene expression of XMEs in the liver and kidneys of male Fisher rats. The effect of the tea extracts on the gene expression of selected genes was also investigated in rat primary hepatocytes in order to validate differences between *in vivo* and *in vitro* effects.

A review on the fate of xenobiotics within the cell and the ability of cells to combat their effects are highlighted in Chapter 2. A short introduction into the history of the herbal teas and there chemopreventive properties is also included. Evidence on the role of polyphenol-enriched extracts of rooibos and two honeybush species on the expression of XME's are presented in Chapter 3, highlighting differences in the responses between the liver and kidneys. The validation of the primary hepatocyte cell culture system as an *in vitro* test model to further predict *in vivo* changes are investigated and differences outlined in Chapter 4. Findings of the thesis are critically reviewed and contextualised with respect to the outcome of the present findings in the General Discussion section (Chapter 5). Experimental details of the primary hepatocyte culture technique utilised, ethical approval of animal experiments conducted, as well as results to validate certain experimental parameters are summarised in the Addendum section. Abstracts of conference attendance and awards received are attached.

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CHAPTER 2

Literature review

Xenobiotics

Introduction to xenobiotics

Interactions between foreign compounds, i.e. xenobiotics, and cells have been extensively studied in both humans and animals (Wang *et al*., 1994; Hu *et al*., 2010; Le Bail *et al*., 1998; Michalopoulos *et al*., 1976; Guo *et al*., 2009). All life forms are constantly being exposed to xenobiotics, beneficial or harmful depending on the dose, which may originate from both naturally occurring and synthetic compounds and are often lipophilic in nature.

Some of these xenobiotics may prove to be advantageous to a cell and can be utilised in normal biological processes to provide energy or act as precursors in the synthesis of macromolecules such as steroids (Wetzel *et al*., 1994). However, xenobiotics such as many environmental chemicals are harmful and may interact with the cell's metabolic pathways and basic cellular constituents such as RNA, DNA (Boyland and Green, 1962) and proteins, leading to the disruption of normal cellular functions (by inducing cell damage and cell death) and ultimately diverse toxic effects.

(i) Environmental carcinogens

It is estimated that the majority of human cancers results from exposure to environmental carcinogens associated with certain life style factors such as diet and tobacco use (Miller, 1978; Wogan *et al*., 2004). These environmental carcinogens are diverse in nature regarding their chemical structures and mode of action which has been studied extensively to evaluate their risk in cancer

development in humans. Depending on their chemical structures these carcinogens can broadly be divided in different categories such as the poly aromatic hydrocarbons (PAH), aromatic and heterocyclic amines, nitrosamines, alkylating agents and certain fungal toxins.

Tobacco carcinogens

Extensive prospective epidemiologic studies clearly establish cigarette smoking as the major cause of lung cancer (Blot and Fraumeni, 1996). Tobacco smoke contains carcinogenic polycyclic aromatic hydrocarbons (PAHs) (Hecht, 1999) and tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-Nitrosonornicotine (NNN), N'-Nitrosoanabasine (NAB) and N'-Nitrosanatabine (NAT) (Figure 1), which are formed from natural components of the tobacco plant. Carcinogens such as NNK and PAHs require metabolic activation to exert their carcinogenic effects; there are competing detoxification pathways, and the balance between metabolic activation and detoxification differs among individuals and will affect cancer risk.

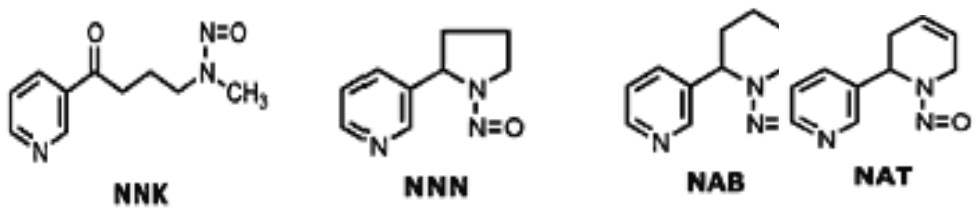


Figure 1 Representation of tobacco-specific nitrosamines.

Diet related carcinogens

Diet has long been recognised as one of the major factors that can influence the development of cancer (Doll and Peto, 1981; WCRF, 1997). Some carcinogens occur naturally on food, such as nuts and grains that are contaminated by mycotoxin-producing fungi. Other carcinogens are formed during food preparation and preservation; polycyclic hydrocarbons, such as benzo[a]pyrene (B[a]P), are formed during cooking of meat on an open flame, and the meat preservative, sodium nitrite, can be converted to nitrosamines.

(ii) Pharmaceutical drugs

Pharmaceutical drugs are considered to be xenobiotics, since it is not produced by the organism neither is it part of its normal diet. These drugs include analgesics (acetaminophen), chemotherapeutics (rifampicin) and antidepressants, amongst others. Drugs share the same metabolic enzymes as other xenobiotics (Block and Gyllenhaal, 2002), therefore interactions between xenobiotics as well as certain cellular processes may occur.

(iii) Plant polyphenols

Polyphenols are essential secondary metabolites produced by plants for protection, pigmentation, growth and pollination (Gould and Lister, 2006) and are considered a xenobiotic if ingested by animals or humans.

Plant polyphenols possess an aromatic ring bearing one or more hydroxyl substituents. The structure of the natural polyphenols varies from simple

molecules, such as phenolic acids, to highly polymerised compounds, such as condensed tannins (Harborne, 1980). Flavonoids represent the most common and widely distributed group of plant phenolics. Their common structure consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Harborne, 1980; Heim *et al*., 2002) (Figure 2).

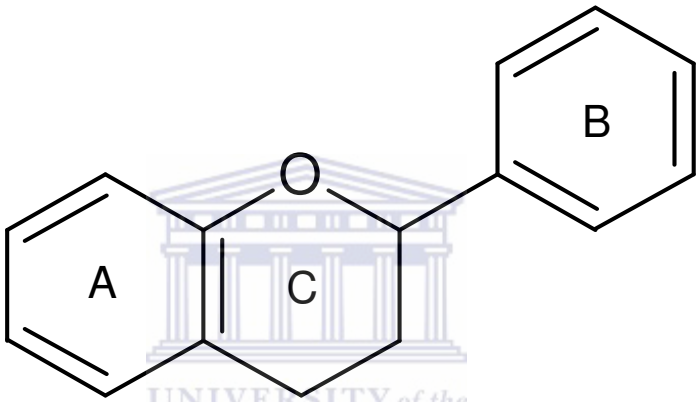


Figure 2 The nuclear structure of flavonoids.

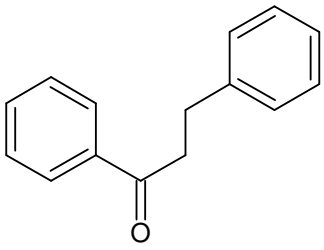
Flavonoids are a large class of naturally occurring secondary metabolites (Huffman, 2003). With more than 6500 different compounds described, these polyphenols are widely present in green plants such as vegetables, fruits and teas (Hollman and Katan, 1997; Harborne and Williams, 2000). Based on different substitutions and oxidation status of the chromane ring (C-ring), flavonoids can be classified into subclasses flavones, flavonols, flavonones, flavanols, isoflavones, chalcones, dihydrochalcones and anthocyanidins (Figure 3). Studies suggest that these flavonoids are metabolised by the same

enzymes (phase I and II) that metabolise the majority of the known pharmaceutical drugs (Zhou *et al.*, 2004).

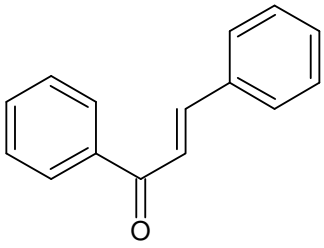
The effect of xenobiotics on cellular metabolic pathways will be discussed.

Metabolism

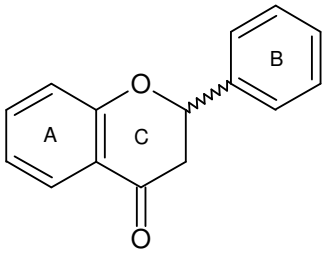
Metabolism can be described as the breakdown (catabolism) and/or build up (anabolism) of molecules in a cell in order to protect it against foreign compounds, or to synthesise macromolecules required for basic biological processes. Metabolic enzymes are crucial to metabolism since it regulates metabolic pathways in response to changes in the cell's environment or signals from other cells. These enzymes are able to metabolise a variety of substrates, which in turn compete for the binding sites of the enzymes. Therefore one substrate can alter the kinetics of the other. Chemicals, such as prescribed pharmaceutical drugs to treat many disease conditions in humans, also interact with the same metabolic pathways which eventually may affect their pharmacological mode of action (Manzi and Shannon, 2005). Several non-nutrient hydrophilic xenobiotic constituents from plant origin that are widely distributed in food also undergo numerous endogenous metabolic events (Galli, 2007). As similar metabolic pathways exist between the different xenobiotics in the cell, several interactions are known to exist which could result in beneficial and/ or adverse cellular effects (Huang and Lesko, 2004).



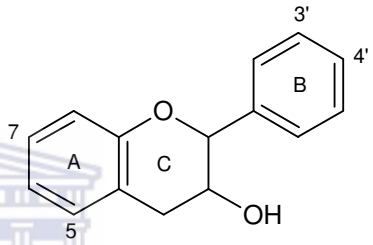
Dihydrochalcone



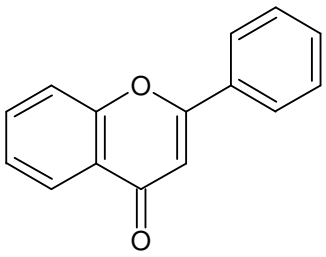
Chalcone



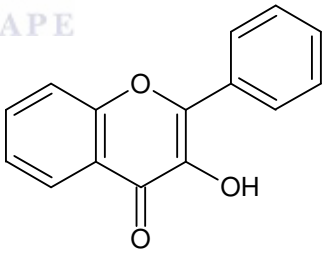
Flavanone



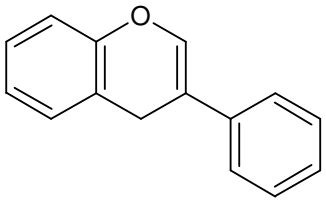
Flavanol



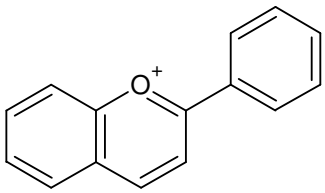
Flavone



Flavonol



Isoflavone



Anthocyanidin

Figure 3 Basic structures of dietary flavonoids (Joubert *et al*., 2008).

The biological basis for xenobiotic metabolism is to decrease the period during which a foreign substance is retained in the body. Essentially the xenobiotic is converted to a water-soluble form (a process known as biotransformation), making it less soluble in the lipid-based membranes facilitating excretion from the cell (Kalra, 2007).

When xenobiotics enter the organism, it is subjected to phase I oxidation, reduction, hydrolysis and hydration reactions which results in a functional group being added or unveiled. Phase II enzymes then conjugate endogenous molecules (glutathione, amino acids, sulfates, acetyl groups, etc) to the functional groups and the product are excreted via the urine, faeces, breath or sweat. Conversely, the metabolism of these compounds can sometimes lead to more reactive intermediary compounds that may exhibit adverse biological effects that exceed that of the parent compound (Lin and Lu, 1997; Guengerich, 2003).

Over centuries, organisms have evolved a complex enzyme system to eliminate foreign/toxic compounds. The enzymes that play a role in xenobiotic metabolism are located in specific cell types in various organs, but are more prominent in the liver (Yamada *et al*., 1997). The sites for biotransformation are summarised in the Table 1.

Table1 Enzyme localisation within the various organs that play a role in the biotransformation of xenobiotics.

Organ	Location of enzymes
Liver	Parenchymal cells (hepatocytes)
Kidney	Proximal tubular cells (S3 segment)
Lung	Clara cells, Type II alveolar cells
Intestine	Mucosa lining cells
Skin	Epithelial cells
Testes	Seminiferous tubules, Sertoli cells

Morphology and function of the liver: Adaptation to xenobiotic metabolism

The liver is the largest gland in the body which constitutes 2-5% of the body weight of an adult male and is the first organ that comes into contact with nutrients and xenobiotics absorbed by enterocytes via the portal vein. Its unique metabolism and relationship to the gastro intestinal tract (GIT) makes it an important site for biotransformation of xenobiotics. The main function of the liver is the production of bile, regulation of plasma proteins and glucose, biotransformation of drugs and toxins, and to detoxify endogenous compounds (Pineiro-Carrero and Pineiro, 2004).

The liver consists of four main types of cells; hepatocytes, endothelial cells, Kupffer cells and stellate cells (Figure 4). The hepatocytes are the biosynthetic engines of the liver. Their prominent golgi system and rough endoplasmic reticulum enable them to synthesise and secrete a variety of enzymes.

Endothelial cells line the sinusoids and serve as a barrier between the blood and hepatocytes. The Kupffer cells function as macrophages and the stellate cells store fat and vitamin A. The hepatocytes are arranged in lobules which are divided into three zones according to its proximity to the portal vein, hepatic artery and hepatic vein (Pineiro-Carrero and Pineiro, 2004). Zone 1 hepatocytes are closest to the portal vein and receive the richest oxygen and nutrient supply and have a high concentration of enzymes involved in cellular respiration; they mostly synthesise glycogen and other proteins. Since zone 3 is located closest to the hepatic veins, little oxygen is available and the hepatocytes are involved in the glycolytic energy production and contain cytochrome P450 (CYP) enzymes. Therefore, the hepatocytes in zone 3 are more specialised in biotransformation reactions. Zone 2 is the intermediate area of hepatocytes between zones 1 and 3; its function depends on how close it is to the portal vein.

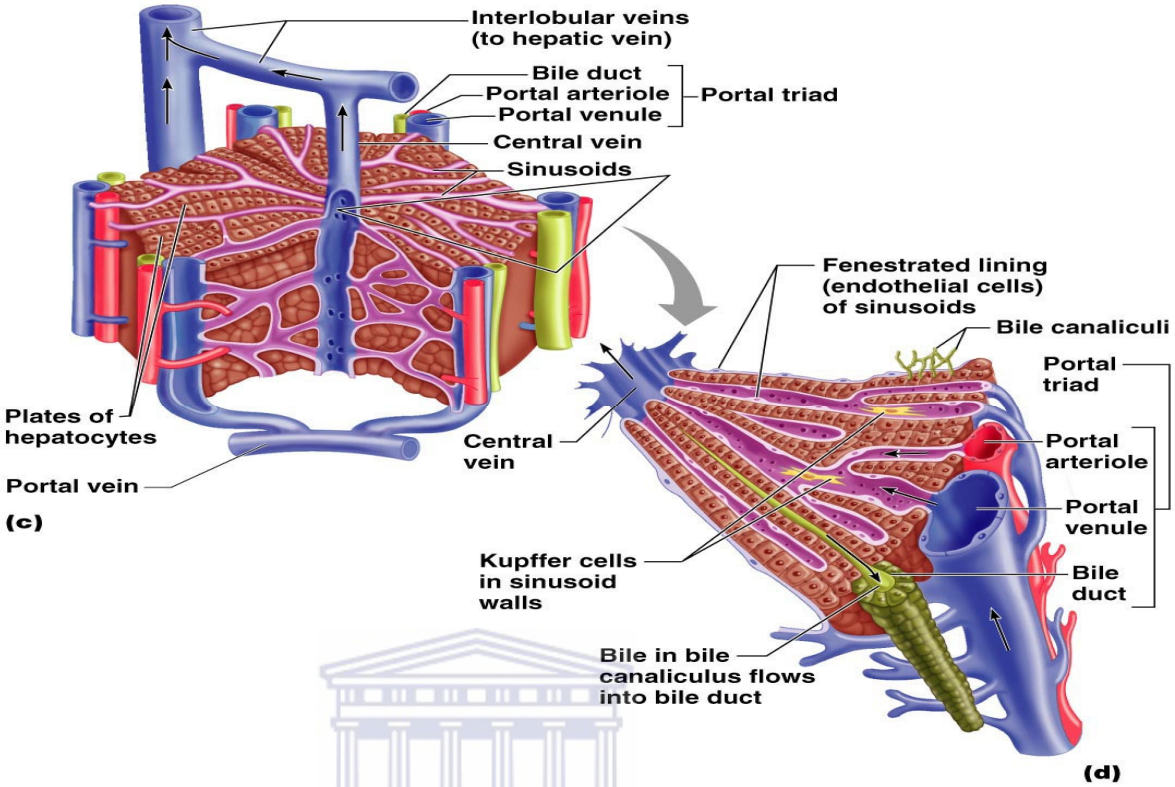


Figure 4 Internal structure of the liver lobule (Pineiro-Carrero and Pineiro, 2004).

Morphology and function of the kidneys: Adaptation to xenobiotic metabolism

The kidneys play a very important physiological role in maintaining water and electrolyte homeostasis, synthesis, metabolism and secretion of hormones, and excretion of the waste products of metabolism. Additionally, the kidneys play a major role in the metabolism and excretion of drugs and other xenobiotics (Anders, 1980; Bock *et al*., 1990).

The kidney is divided into two main areas: the outer renal cortex and the inner renal medulla. Within the medulla there are cone-shaped renal pyramids separated by renal columns. Each kidney consists of about one million

microscopic nephrons, the most basic structures of the kidneys. Nephrons are composed of the glomerulus inside the Bowman's capsule, the Malpighian corpuscle, proximal and distal convoluted tubules, and the loop of Henlé. Using an isolated tubule preparation in rats and rabbits, Endou (1983) found that CYP450 was localised to the proximal tubule with the highest concentration in the microsomes of the S2 and S3 segments (Figure 5). Cytochrome P450 enzyme activities have been found in the kidneys.

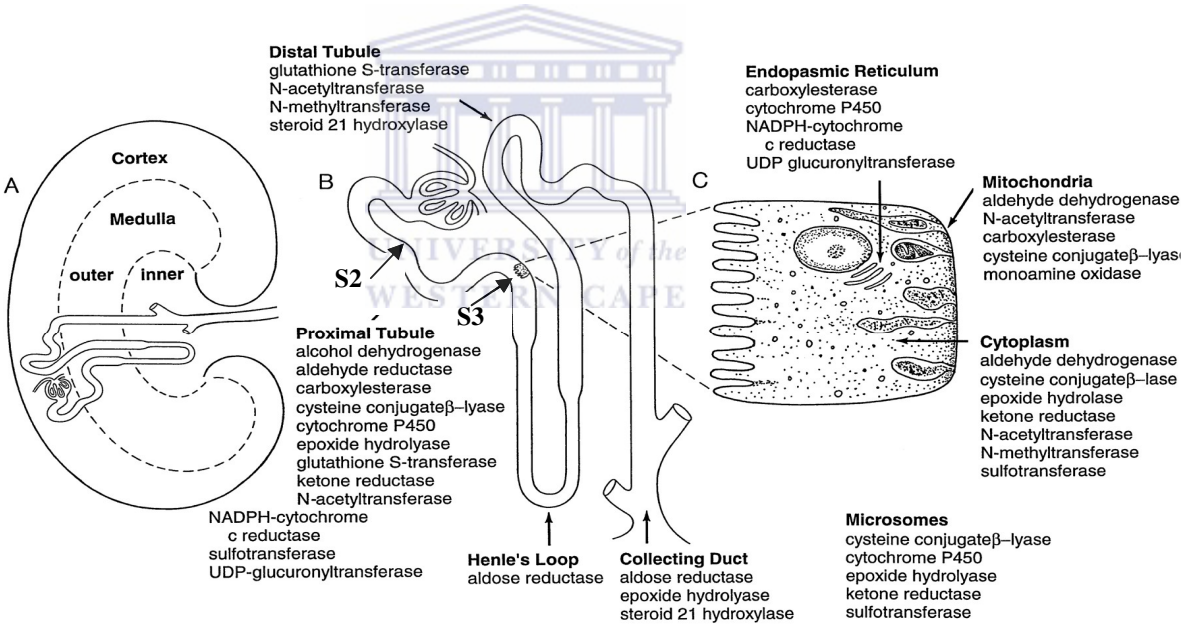


Figure 5 Regional (A), tubular (B), and subcellular (C) localisation of xenobiotic-metabolising enzyme systems in the kidney (Lohr *et al.*, 1998).

Phases of xenobiotic metabolism

The detoxification enzymes are classified according to phase of action: (i) phase I (cytochrome P450 monooxygenases), (ii) phase II (UDP-glucuronosyltransferases, glutathione-S-transferases and sulfotransferases) and (iii) phase III (ABC transporters). These detoxification systems are highly complex and responsive to the individual's environment, lifestyle and genetic uniqueness.

(i) Phase I metabolism

Phase I reactions involve the chemical modification of xenobiotics by introducing or unveiling polar functional groups that provide sites for phase II metabolism. These include oxidation, reduction and hydrolysis reactions. Phase I enzymes are responsible for adding, or exposing, a functional group to xenobiotic molecules by adding one oxygen atom leading to the availability of a polar functional group such as -OH, -SH, $-NH_2$, or –COOH in the molecule undergoing biotransformation. At this stage, the molecule is slightly more hydrophilic, but not yet ready for excretion. Further metabolism occurs via phase II reactions.

The principal phase I enzymes include the superfamily of haem-protein mixed-function oxidases (cytochrome P450), flavin monooxygenases, monoamine oxidases, esterases, amidases, hydrolases, reductases, dehydrogenases and oxidases. The cytochrome P450 supergene family of enzymes is generally the

first enzymatic defense against foreign compounds. They also play a pivotal role in the biosynthesis and catabolism of endogenous substrates such as bile salts, vitamins, fatty acids, prostaglandins and steroids.

Discovery, evolution and nomenclature of cytochrome P450

Cytochrome P450 was first isolated in the 1950s. A cellular pigment in rat and pig microsomes was discovered when an unusual, red-shifted, visible absorption maximum at about 450nm was observed (Klingenberg, 1957; Garfinkel, 1958). This pigment at 450nm was observed when the reduced form of the enzyme formed a complex with carbon monoxide. This absorption maximum was quite different from that displayed by typical haemproteins such as myoglobin (435nm). The name cytochrome P450 can be explained as “cyto” meaning microsomal vesicle, “chrome” for coloured, “P” for pigmented and “450” for the 450nm spectrophometric absorption peak (Ortiz de Montellano, 1995). In 1958 it was found to be labile to detergents, low pH and enzymatic digestion. It was further characterised and purified by Omura and Sato (1964) and was shown to be a b-type cytochrome (Omura and Sato, 1962). In 1965 cytochrome P450 was established as being the terminal oxidase of the liver microsomal drug-metabolising enzyme system (Cooper *et al.*, 1965). The discovery that high concentrations of glycerol stabilise CYPs against detergent treatment was made by Ichikawa and Yamano (1967). This was a major breakthrough in the purification of membrane-bound P450s.

Various forms of cytochrome P450 were successfully identified in the late 1970s, followed by the cloning of their cDNAs in the 1980s. This showed the presence of different molecular species in animals, plants and fungi where each cytochrome P450 enzyme exhibit very distinct but broad substrate specificity. The highly conserved amino acid sequence motifs of those isoforms were indicative of their evolutional divergence from a single ancestral form (Nelson and Strobel, 1987; Gotoh, 1989). Individual forms are important in the metabolism of foreign hydrophobic compounds which may include dietary components, xenobiotics, therapeutic drugs (eg. phenobarbital, PB) or chemical carcinogens (Nebert and Gonzales, 1987). Many isoforms play vital roles in some tightly regulated pathways, such as developmental regulation and modulation by steroid or polypeptide hormones. The classification system of all P450s was based on the degree of similarity in their amino acid sequences. In 1987 isoforms were divided into “families” and “subfamilies”. Members within the same family typically have >40% identity in their amino acid sequence, whilst members of the same subfamily are at least >55% identical (Nebert, 1989, 1991; Nelson, 1993; Guengerich, 1997). It is estimated that mammals have about 50-80 P450 genes in its genome and plants might have even more. However, no P450 genes have been found in the genome of *Escherichia coli* (Nelson, 1999). In humans 57 functional CYP genes and 58 pseudogenes have been sequenced (Nelson, 2002; Purnapatre *et al*., 2008). These comprise 18 families and 44 subfamilies. Families CYP1-4 are mainly involved in xenobiotic metabolism. The contribution of the major isoforms to overall drug metabolism

is as follows: 1A2 (4%), 2A6 (2%), 2C9 (10%), 2C19 (2%), 2E1 (2%), 2D6 (30%) and 3A4 (50%) (Purnapatre *et al*., 2008). Others are typically not involved in xenobiotic metabolism, but rather metabolise physiological endogenous substances e.g. CYPs 11, 17, 19 and 21 catalyse the hydroxylation required for steroid hormone biosynthesis from cholesterol (Nelson *et al*., 1996). In rats, however, 60 genes and 4 pseudogenes have been sequenced.

The isolation and characterisation of the cytochrome P450 system led to the current understanding of their mechanism of action (Estabrook, 2003).

Cytochrome P450 isoforms

The CYP families 1-3 are responsible for 70-80% of all phase I dependent drug metabolism (Wijnen *et al*., 2007).

CYP1

The CYP1 family includes two sub-families, CYP1A and CYP1B, which consists of isoforms 1A1, 1A2 and 1B1, respectively (Nebert and McKinnon, 1994). CYP1A is linked to the aryl hydrocarbon receptor (AhR) as inducible genes (Gonzales *et al*., 1993; Dogra *et al*., 1998; Waxman, 1999) and the enzyme is capable of bioactivating several procarcinogens (Heidel *et al*., 2000). CYP1A1 activates B[a]P and other polyaromatic hydrocarbons while CYP1A2 activates aromatic amines, such as 2-acetylaminofluorene, heterocyclic amines and aflatoxin B_1 (Eaton *et al*., 1995; Gallagher *et al*., 1996; Pelkonen and Raunio, 1997; Rendic and Di-Carlo, 1997). CYP1A1 is expressed in many tissues, but

typically only after induction with TCDD or other AhR ligands. CYP1A2 is found exclusively in the liver (Dogra *et al*., 1998) and is primarily involved in the hydroxylation and demethylation of compounds through oxidative metabolism. Its substrates include caffeine (Miners and Birkett, 1996), PAH (Castorena-Torres *et al*., 2005), theophylline (Rasmussen and Brosen, 1997) and naproxen (Miners *et al*., 1996). Grapefruit juice (GFJ) (Fuhr *et al*., 1993; Tassaneeyakul *et al*., 2000) and drugs like fluvoxamine (Becquemont, *et al*., 1998) and ciprofloxacin (Fuhr *et al*., 1990) are potent inhibitors of CYP1A2. This inhibiting action increases serum levels of substrates resulting in toxicity. Examples of enzyme inducers are cruciferous vegetables, charbroiled meat (Wijnen *et al*., 2007) and tobacco smoke (Wardlaw *et al*., 1998; Schrenk, *et al*., 1998; Zevin and Benowitz, 1999). CYP1A2 also metabolises PAHs into carcinogenic compounds and has a higher activity in Chinese men (Ou Yang *et al*., 2000).

CYP1B1 is a more recently characterised member of the CYP1 family. It is constitutively expressed in most tissues, but is also inducible through the Ah receptor pathway (Cheung *et al*., 1999). However, the protein is undetectable in normal human liver (McFadyen *et al*., 2004). CYP1B1 typically metabolises endogenous estrogens (Hayes *et al*., 1996), as well as activating the biotransformation of heterocyclic amines found in charcoal-broiled meat (Crofts *et al*., 1997). The presence of CYP1B1 has been proven to decrease the sensitivity of cancer cells to the cytotoxic effects of a specific anticancer drug (docetaxel) making it less cytotoxic (McFadyen *et al*., 2001). Polymorphisms of

CYP1B1 gene at codons 119 and 432 has been found to be a risk factor for renal cell cancer (Sasaki *et al*., 2004).

CYP2

The CYP2E1 gene is constitutively expressed in the liver and the enzyme accounts for 7% of total CYP content. It catalyses highly conserved functions, including the oxidation of the fatty acid, arachidonic acid (Capdevila *et al*., 1992; Laetham *et al*., 1993). The enzyme is also involved in gluconeogenesis through its ability to metabolise acetone to acetol (Lieber, 1997). However, its primary functions are to metabolise ethanol and acetaminophen (paracetamol), and to metabolically activate many low molecular weight compounds such as *N*-nitrosamines, aniline, vinyl chloride, and urethane (Bartsch and Montesano, 1984; Guengerich *et al*., 1991) in an attempt to eliminate it. Polymorphism of CYP2E1 is very common, resulting in serious complications such as cancer and toxicity.

CYP3A

The CYP3A family is the most abundant cytochrome P450 enzymes in the liver, accounting for 30% of total hepatic content and 70% of intestinal wall content (Chang and Kam, 1999). The expression of CYP3A seems to be regulated by pregnane X receptor (PXR), a nuclear receptor belonging to the family of ligand-activated (Kliewer *et al*., 1998) transcription factors. PXR binds as a heterodimer with 9-cis retinoic acid receptor (NR2B) to DNA response elements in the regulatory regions of CYP3A gene (Kliewer *et al*., 2002). CYP3A does not

exhibit genetic polymorphism, but its isoforms are expressed differently across species; the rat isoform is CYP3A1/23. The human isoform CYP3A4 is known to metabolise approximately 60% of prescribed drugs (Guengerich, 1999). The enzyme is induced by rifampicin (Li and Chiang, 2006) and the coding gene has been shown to be down-regulated by grapefruit juice (Garteiz *et al*., 1982). Although metabolism of exogenous compounds is the major function of this isoform, it is also able to metabolise endogenous compounds such as progesterone, estradiol, testosterone and cortisol (Waxman, 1996).

CYP structure and mechanism of action

The microsomal cytochrome P450 system is composed of two enzymes i.e. the haem-containing enzyme CYP450 and NADPH-cytochrome P450 reductase (Lu and Coon, 1968; Masters *et al*., 1971). These enzymes are present in all organs, but predominantly in the liver, entrenched in the phospholipid matrix of the endoplasmic reticulum and the mitochondria (Omura, 1999). It is the phospholipid layer that facilitates the interaction between the two enzymes. The active site is deep within the enzyme, next to a catalytic iron atom held at the center of a haem group. It can accommodate a variety of different carbon-rich molecules, pressing them tightly against the active oxygen atom (Goodsell, 2001). The reaction begins with the introduction of a single atom of molecular oxygen derived from O_2, into the substrate, with the simultaneous reduction of the accompanying oxygen atom to water.

The general equation for CYP catalysis is shown below:

$$RH^* + O_2 + H^+ + NADPH \xrightarrow[\text{CYP450}]{} ROH + H_2O + NADP^+$$

*RH represents the substrate and ROH represents the oxidised product.

This equation only represents a very general and simple mechanism. The reaction is best represented as a cyclic reaction showing the interactions that occur between cytochrome P450 and the NADPH-cytochrome P450 reductase (Figure 6) (Porter and Coon, 1991; Guengerich 1991; Guengerich *et al*., 1998). The reaction is initiated when the substrate binds to the active site of the cytochrome P450, close to the haem bound region of the molecule. It involves the reduction of molecular oxygen by the stepwise addition of two electrons. These electrons are donated by the reducing co-factor NADPH. NADPH is a two-electron donor but cytochrome P450 can only accept one electron at a time. Therefore an accessory enzyme is required such as NADPH-cytochrome P450 reductase which possesses two flavin prosthetic groups (Suzuki, 1965). This catalyses the transfer of two electrons from NADPH to the P450 (Omura *et al*., 1966). The ratio of NADPH-cytochrome P450 reductase to CYP molecules present in liver microsomes is about 2:1. It is during the first electron donation that the iron atom present in the CYP is reduced to the ferrous state (Fe^{2+}) and molecular oxygen binds to the CYP complex. A second electron is then donated

to the CYP from the NADPH-cytochrome P450 reductase (Iyanagi, 2005). This is followed by electron arrangement, insertion of an oxygen atom and product release with the generation of water or hydrogen peroxide

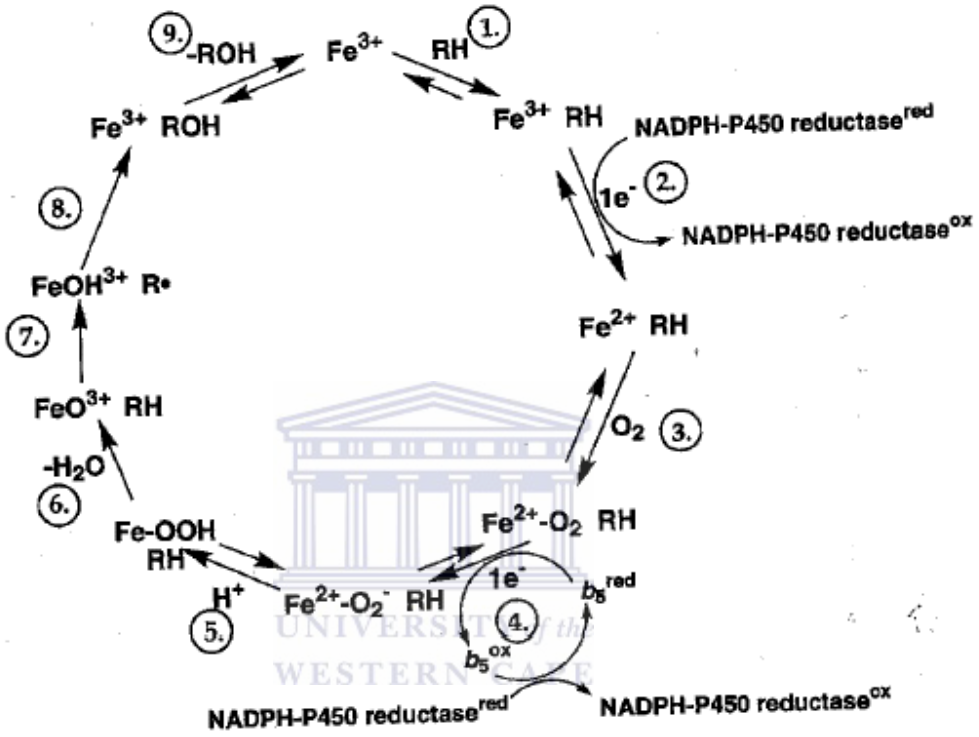


Figure 6 Generalised catalytic cycle for P450 reactions (Guengerich, 1991 and Guengerich *et al*., 1998).

CYP1 family members are under the transcriptional regulation of the AhR (Dogra *et al*., 1998; Tredger *et al*., 2002; Baumgart *et al*., 2005). AhR binds exogenous molecules such as PAHs present in air pollution and cigarette smoke (Song *et al*., 2002; Denison *et al*., 2003). Upon binding, AhR translocates to the nucleus where it binds aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT dimer regulates transcription of target genes (Denison *et al*., 2003).

The regulation of drug metabolism is highly complex, being affected by both genetic and non-genetic host factors. Genetic factors may cause variability in an enzyme's activity, function, stability and responsiveness to an inducer or regulator (Kalow, 2001). These variations in the metabolism of a drug may affect the responsiveness of a patient to a drug and even predispose the patient to adverse effects (Park and Pirmohamed, 2001). A single base change can have dramatic effects on specificity. A study on mutagenesis showed that a substitution of a single amino acid, S473V, allows CYP2C2 to accept progesterone as a substrate, whereas CYP2C2 is normally a lauric acid hydroxylase (Ramarao and Kemper, 1995). This change in substrate specificity is very important because it now allows this enzyme to be involved in steroid metabolism.

Non-genetic factors such as sex, age, xenobiotics and disease state can also affect the expression of CYPs. Cytochrome P450 is important to our species individuality because no two species will have the same complementary P450 genes (Nelson, 1999).

Xenobiotic induction/inhibition of cytochrome P450

Xenobiotics metabolising enzymes can either be induced or inhibited by concomitantly administered drugs, food substances or pollutants, which can result in clinically significant interactions (Pirmohamed and Orme, 1998). Enzyme induction results in an increased level and activity of the enzyme (reviewed by Berthou, 2001). The most commonly prescribed enzyme inducers

are listed in Table 1 (Addendum I), together with drugs that can be affected (Pirmohamed, 2008). An example of an enzyme induction interaction is warfarin, an anticoagulant, and carbamazepine, an anticonvulsant. Co-administration of these two drugs will lead to an increase in metabolism of warfarin, which could lead to a failure of anticoagulation. Enzyme induction has also been observed with the herbal medicine St John's wort (SJW), which affects the metabolism of warfarin as well as immunosuppressant and anti-viral drugs (Mannel, 2004).

Inhibition of CYP enzymes lead to a decrease in the metabolic rate, resulting in an increase in drug concentration and consequent toxicity. A list of CYP enzyme inhibitors is shown in Table 2 in the addendum I (Pirmohamed, 2008). An example of an inhibitor is grapefruit juice (GFJ) which inhibits CYP3A4. Grapefruit juice interacts with a number of drugs including terfenadine, ciclosporin and simvastatin (Ameer *et al*., 1997). Knowledge of the interaction potential of herbs and drugs may prevent adverse drug interactions.

Steroidogenesis: the role of cytochrome P450

The role of CYPs in steroid hydroxylation was first elucidated by Estabrook *et al.,* (1963). Six forms of cytochrome P450 and two hydroxysteroid dehydrogenases are the main enzymes involved in steroid biosynthesis (Omura and Morohashi, 1995) from cholesterol in animal tissues (Nelson *et al*., 1996). The very first steroidogenic cytochrome P450 purified was P450scc in 1973

(Shikita *et al*., 1973). This was followed by five more ($P450_{11\beta}$, $P450_{aldo}$, $P450_{17\alpha}$, $P450_{c21}$ and $P450_{arom}$). P450scc, $P450_{11\beta}$, $P450_{aldo}$, are located in the inner membrane of the mitochondria, whereas $P450_{17\alpha}$, $P450_{c21}$ and $P450_{arom}$ are in the membrane of the endoplasmic reticulum. The two hydroxysteroid dehydrogenases (HSD), 3β-HSD and 17β-HSD, are also associated with the endoplasmic reticulum membrane (Omura and Morahashi, 1995). These CYPs together with the two HSDs regulate the biosynthesis of steroids (Figure 7).

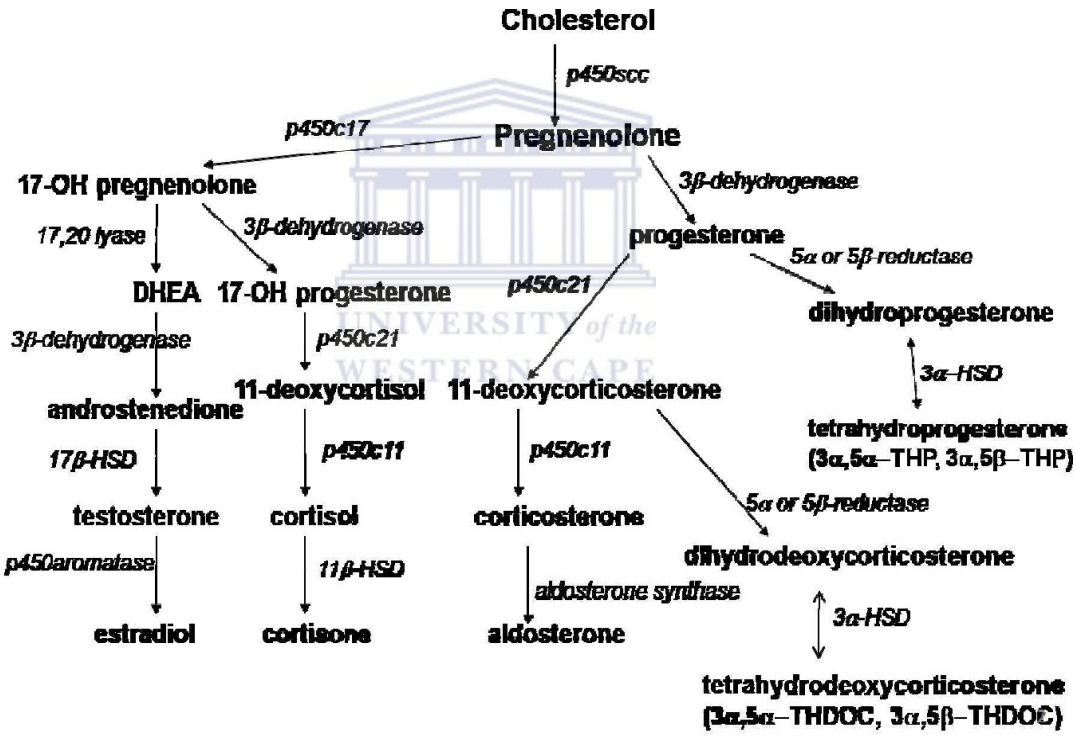


Figure 7 Enzymes involved in steroid biosynthesis.

The relationship between cytochrome P450 and cancer

The natural chemical environment contains many toxic compounds, such as procarcinogens and direct carcinogens that may require metabolism to exert an effect. Procarcinogens are parent compounds that cause cancer following activation via normal metabolism, e.g. benzo(a)pyrene. Direct carcinogens refer to the reactive metabolite that covalently binds to and modify DNA. Cytochrome P450 plays an important role in the aetiology of cancer diseases (Oyama *et al*., 2004; Rooseboom *et al*., 2004) by bioactivating carcinogens e.g. aflatoxin $B_{1,}$ a hepato carcinogenic mycotoxin. Aflatoxin B_1 is metabolised by CYP1A1 to an exo epoxide, which binds and intercalate into the DNA resulting in DNA adducts and mutations (Guengerich, 2006).

Cytochrome P450 as a target for anticancer therapy

The CYP superfamily plays a central role in influencing the response of tumors to anticancer drugs. They can either activate or deactivate it. Drug activation (resulting in cytotoxicity) and deactivation (resulting in potential resistance) depend on the relative amount and activity of specific CYPs in individual tumor cells (McFadyen and Murray, 2005). CYP1B1 has been identified as the main CYP present in a variety of human cancers, in various tissue types e.g. prostate cancer cells (Beuten *et al*., 2000) and lung carcinoma cells (Wenzlaff *et al*., 2005). Although CYP1B1 mRNA appears to be expressed in a wide variety of normal tissues, the 1B1 protein has not yet been detected (Church *et al*., 2010). CYP1B1, along with 1A1, has the ability to metabolise anticancer-drugs, a

reaction that might help tumors to avoid chemotherapeutic induced cytotoxicity (McFadyen *et al*., 2001; Stiborova *et al*., 2001; Patterson and Murray, 2002). In addition to this, CYP1B1 also activates several carcinogens in chemical classes of polycyclic aromatic hydrocarbons, heterocyclic amines, aromatic amines, and nitropolycyclic hydrocarbons. Of importance, however, is the role of CYP1B1, as well as that of CYP19A1, in estradiol metabolism. CYP1B1 catalyses the hydroxylation of estradiol primarily at the C4-position, which plays an important role in estrogen-related tumorigenesis, whilst CYP19A1 (aromatase) catalyses the rate limiting step in the conversion of androgens to estrogens (Bruno and Njar, 2007). A study using CYP1B1 knockout mice showed strong resistance to 7,12-dimethylbenz[a]anthracene (DMBA) induced tumor formation. Another study by Heidel *et al*., showed that CYP1B1 deletion prevents profound cytotoxicity in bone marrow, produced by DMBA (Heidel *et al*., 2000; Galvan *et al*., 2003). A possible strategy for chemoprevention would therefore be to inhibit transcription or translation of these genes or enzymes.

(ii) Phase II metabolism

Phase II of biotransformation involves conjugation reactions that result in inactivation or detoxification of xenobiotics. However, it can also result in bioactivation or reactive species generation (Cantelli-Forti *et al*., 1998). Studies have shown that glutathione-S-transferase (GST) activates halogenated hydrocarbons and epoxide hydrolases while sulfotransferases activates PAHs (Wood *et al*., 1976; Kasper and Henion, 1980; Wolf *et al*., 1984; Hanna *et al*.,

1992). Phase II reactions typically occur much faster than phase I reactions, making the latter the rate limiting step of biotransformation. Most phase II enzymes are located in the cytosol, except UDP-glucuronyltransferases (UGTs), which are microsomal. Their substrates are typically lipophilic and access the intracellular space by diffusion (Zamek-Gliszczynski *et al*., 2006). Phase II enzymes conjugate large molecules onto chemical substrates, such as carcinogens that contain free amine, hydroxyl or carboxyl groups. The conjugated moieties makes the carcinogen-conjugate charged and thus more water soluble, easy to excrete and less likely to be reabsorbed. It is important to note that phase I and II xenobiotic metabolising enzymes are able to activate or detoxify xenobiotics simultaneously, depending on the catalysed chemical reaction (Guengerich, 1985).

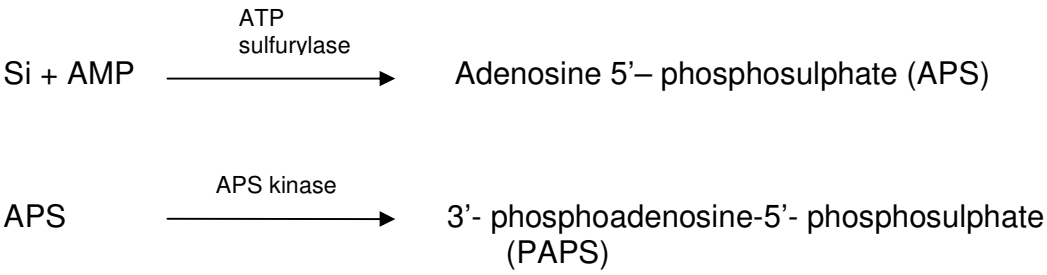
Glucuronidation

Glucuronidation, catalyzed by a family of membrane-bound UGT enzymes, is a metabolic reaction converting lipophilic xeno- and endobiotics to more water soluble metabolites, the glucuronides (Burchell and Coughtrie, 1989; Miners and Mackenzie, 1991). The reaction occurs primarily in the liver, intestinal mucosa and kidneys. UGTs transfer glucuronic acid from UDP-glucuronic acid to the aglycone substrate which includes compounds containing nucleophilic functional groups such as alcohols, phenols and primary and secondary amines. The clearance of some endogenous substances, such as bilirubin, steroid hormones, and bile acids, is largely dependent on UGT-mediated

metabolism. In addition, a large variety of drugs, such as the carboxylic acid drug naproxen, are metabolised primarily by glucuronidation (Kaivosaari, 2010). Aspirin, menthol, synthetic vanilla, flavonoids (Otake *et al*., 2002), food additives such as benzoate, and some hormones are also metabolised by UGTs.

Sulfation

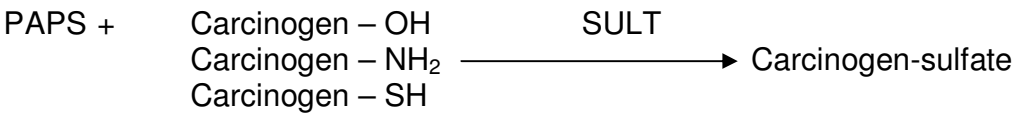
Sulfation increases molecular hydrophilicity of xenobiotics in preparation for biliary excretion or efflux across the hepatic basolateral membrane for subsequent renal clearance (Zamek-Gliszczynski, 2006). Intracellular inorganic sulfate originates from hydrolysis of sulfoconjugates, oxidation of reduced organic sulfur, or uptake of sulfate from blood across the basolateral membrane (Markovich, 2001). The following reaction occurs in the cytosol:



Si= inorganic sulfur; AMP= Adenosine monophosphate. PAPS is the donor of sulfate groups (SO_4^-) in all sulfate conjugation reactions (Low, 1998; Klaassen and Boles, 1997).

Sulfotransferases (SULTs) are most abundant in the liver, but are found in a variety of tissues. They are generally high-affinity, low- capacity biotransformation enzymes that operate effectively at low substrate concentrations (Sacco and James, 2005).

The general reaction of SULT can be represented by the equation below:



PAPS - 3'- phosphoadenosine-5'-phosphosulphate; SULT -Sulfotransferases

Soluble or cytosolic SULT enzymes are subdivided into three families: SULT1 (phenol sulfotransferase family), SULT2 (hydroxysteroid sulfotransferase family) and brain-specific SULT4 (Weinshilbaum *et al*., 1997; Falany *et al*., 2000). The SULT1 family is further subdivided into four subfamilies: SULT1A (phenol-type xenobiotics), SULT1B (dopa/tyrosine and thyroid hormones), SULT1C (hydroxyarylamines) and SULT1E (estrogens). Although individual SULT isoenzymes have substrate preference, there is evidence of substrate overlap (Nagata *et al*., 1993; Doerge *et al*., 2000). SULT plays an important role in the metabolism of steroids, thyroid hormones and bile acids (Gamage, 2006). However, it may also result in bioactivation of xenobiotics (Glatt, 2000).

Glutathione conjugation

Glutathione (GSH), a major nucleophile in the cell, is composed of three amino acids (cysteine, glutamic acid and glycine). GSH is specifically directed to scavenge toxic electrophiles and they are also important antioxidants. This combination of detoxification and free radical protection results in glutathione being one of the most important anticarcinogens and antioxidants. Therefore, a deficiency can result in serious liver dysfunction and damage. Although

glutathione is able to spontaneously react with electrophiles, this reaction requires a catalyst. Glutathione-S-transferases (GSTs) are a series of cytosolic enzymes (Ryle and Mantle, 1984; Strange *et al*., 2001) able to catalyse these reactions. GSTs are expressed in all life forms (Strange *et al*., 2000). Conjugation of GSH typically occurs in the cytoplasm of the liver and the conjugates can be excreted intact into bile, or converted to mercapturic acids by the kidney (Anders, 1980) and excreted in the urine (Barnes *et al*., 1959; Boyland and Chasseaud, 1969).

Methylation

Methylation involves conjugating methyl groups to toxins. Most of these methyl groups originate from s-adenosylmethionine (SAM) (Axelrod and Tomchick, 1958). SAM is synthesized from the amino acid methionine, a process which requires the nutrients choline, vitamin B_{12} and folic acid. With the exception of nicotine methylation that produces quaternary ammonium ions, methylation decreases water solubility of drugs. This is mediated by three groups of enzymes: O, N, and S- methyltransferases. O-methylation via catechol-O-methyltransferase (COMT) is important in the metabolism of (1) catecholamines that serve as neurotransmitters (epinephrine, norepinephrin and dopamine) and (2) plant flavonoids containing catechol groups (Manach *et al*., 1999; Nagai *et al*., 2004).

Acetylation

N-acetylation is a major route of biotransformation of drugs containing an aromatic amine (R-NH_2) or a hydrazine group (R-NH-NH_2) (Payton *et al*., 1999; Grant *et al*., 1992). It is dependent on thiamine, panthothenic acid and vitamin C and is catalysed by N-acetyltransferases and requires acetylcoenzyme A (acetyl-CoA) as a cofactor (King and Glowinski, 1983). Humans express only two acetyltransferases, NAT1 and NAT2 (Grant *et al*., 1992).

Amino acid conjugation

Several amino acids (glycine, taurine, glutamine, arginine and ornithine) are used to conjugate toxins. Of these, glycine is the most commonly utilised in phase II amino acid detoxification (Liska, 1998). There are two pathways by which drugs can be conjugated with amino acids. One involves carboxylic acid groups; the other requires an aromatic hydroxylamine group.

(iii) Phase III metabolism

Since some conjugates are generally too large and too polar to diffuse passively to bile or urine, specific transporters are required to aid their movement across cell membranes. The ATP-binding cassette (ABC) transporter superfamily genes represent the largest family of transmembrane proteins. These proteins bind ATP and use energy to drive the translocation of a wide variety of substrates across the extra- and intracellular membranes, including metabolic products, lipids and sterols, and drugs. Transporters

specifically recognise organic acids (glucuronides, sulfates, etc) or organic bases (amines) and pump them from the cell.

Phase III drug transporters play an important role in reducing drug absorption and enhancing drug elimination back to the gut lumen.

Phylogenetic analysis places the 48 known human ABC transporters into seven distinct subfamilies of proteins. A list of ABC transporters and their functions are summarised in Table 2.

Herb-drug interactions

It is imperative to note that many medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another. All ingested substances have the potential to interact with one another, the same transporter and the same metabolising enzymes. Concurrent use of herbs may mimic, magnify or oppose the effect of drugs (Fugh-Berman, 2000). Herb-drug interactions may occur through two major mechanistic routes; inhibition of enzyme action or activation of gene transcription (Plant, 2007). Many studies focus on their effects on xenobiotic metabolising enzymes alone however, Interference at the level of ABC transporters could also play a role. P-glycoprotein (P-gp), an ABC transporter (ABCB1), plays a physiological role in detoxification of toxic metabolites. It secretes these toxic compounds into bile, urine and the intestinal lumen. This function can either be beneficial in the intact organism by preventing substances from being absorbed into the body or negative in the case of the treatment of malignant tumours with cytotoxic anticancer drugs,

which need to enter the cells for their pharmacological effect. P-gp is expressed in natural barriers such as the intestinal epithelium, the blood-brain barrier, liver, and kidney for excretion of xenobiotics. P-gp presents high transport capacity and broad substrate specificity. It generally transports hydrophobic, amphipathic molecules. Some P-gp substrates are able to either inhibit or induce P-gp-mediated transport of other substrates. Herbal constituents have been reported to modulate P-gp expression and/or activity (Romiti *et al*., 1998; Sadzuka *et al*., 2000; Anuchapreeda *et al*., 2002; Bhardwaj *et al*., 2002; Jodoin *et al*., 2002; Kim *et al*., 2003; Choi *et al*., 2003; Zhang and Morris, 2003; Limtrakul *et al*., 2004; Mei *et al*., 2004; Nabekura *et al*., 2005). Constituents of grapefruit and orange juice were also found to block P-gp function (Takanaga *et al*., 2000; Xu *et al*., 2003; Zhou *et al*., 2004). Expression levels of P-gp and xenobiotic metabolising enzymes appear to be regulated by nuclear receptors like pregnane X receptor (PXR) (Savas *et al*., 1999), constitutive androstane receptor and vitamin D binding receptor (Xu *et al*., 2005).

Table 2 List of human ABC genes and their function (Dean, 2002).

Symbol	Alias	Function
ABCA1	ABC1	Cholesterol efflux onto HDL
ABCA2	ABC2	Drug resistance
ABCA3	ABC3/ABCC	Surfactant secretion?
ABCA4	ABCR	*N*-Retinylidiene-PE efflux
ABCA5		
ABCA6		
ABCA7		
ABCA8		
ABCA9		
ABCA10		
ABCA12		
ABCA13		
ABCB1	PGY1/ MDR	Multidrug resistance
ABCB2	TAP1	Peptide transport
ABCB3	TAP2	Peptide transport
ABCB4	PGY3	PC transport
ABCB5		
ABCB6	MTABC3	Iron transport
ABCB7	ABC7	Fe/S cluster transport
ABCB8	MABC1	
ABCB9		
ABCB10	MTABC2	
ABCB11	SPGP	Bile salt transport
ABCC1	MRP1	Drug resistance
ABCC2	MRP2	Organic anion efflux
ABCC3	MRP3	Drug resistance
ABCC4	MRP4	Nucleoside transport
ABCC5	MRP5	Nucleoside transport
ABCC6	MRP6	
ABCC7	CFTR	Chloride ion channel
ABCC8	SUR	Sulfonylurea receptor
ABCC9	SUR2	K(ATP) channel regulation
ABCC10	MRP7	
ABCC11		
ABCC12		
ABCD1	ALD	VLCFA transport regulation
ABCD2	ALDL1/ALDR	
ABCD3	PXMP1/PMP70	

Symbol	Alias	Function
ABCE1	OABP/RNS4I	Oligoadenylate binding protein
ABCF1	ABC50	
ABCF2		
ABCF3		
ABCG1	ABC8, White	Cholesterol transport?
ABCG2	ABCP, MXR, BCRP	Toxin efflux, drug resistance
ABCG4	White2	
ABCG5	White3	Sterol transport
ABCG8		Sterol transport

Effect of flavonoids on xenobiotic metabolisers

A number of studies have shown that flavonoids modulate the CYP450 system, *in vitro* and *in vivo* (Wattenberg *et al*., 1968; Wood *et al*., 1986; Ho and Saville, 2001). Genistein, the soy isoflavone, is metabolised by and decreases the activity of CYP1A1, 1A2, 2E1 and SULT1E1 (Helsby *et al*., 1998; Chan and Leung, 2003; Chen *et al*., 2004; Ohkimoto *et al*., 2004) and increases the activity of UGT (Sun *et al*., 1998) and GST (Ansell *et al*., 2004). Quercetin increases the activity of CYP1A1 (Ciolino *et al*., 1999), UGT (Sun *et al*., 1998; van der Logt *et al*., 2003) and quinone reductase (QR) (Uda *et al*., 1997) and it decreases the activity of CYP1A2 (Tsyrlov *et al*., 1994), 3A4 (Obach, 2000), UGT1A1 (van der Logt *et al*., 2003; Sun *et al*., 1998), GSTP1-1 (van Zanden *et al*., 2003), SULT1A1 (Eaton *et al*., 1996; Ghazali and Waring, 1999; Harris *et al*., 2004) and SULT1E1 (Otake *et al*., 2000; Ohkimoto *et al*., 2004). Additional reactions are summarised in Table 3 in the addendum I.

Effect of herb-drug interactions on chemoprevention and protection

Up to 90% of all cancers are thought to be due to environmental carcinogens (Kwak *et al*., 2001) such as those found in cigarette smoke, food, water and air. High levels of exposure to carcinogens, coupled with slow or absent detoxifying enzymes, significantly increase susceptibility to cancer. Many chemicals require metabolic activation to electrophilic intermediates to exert carcinogenic activity (Miller and Miller, 1983). If not detoxified, these reactive species target nucleophilic groups in DNA, causing point mutations and other genetic lesions. This may result in activation of pro-oncogenes and inactivation or loss of tumour suppressor genes (Kwak *et al*., 2001).

The ability of a carcinogen to interact with its target depends on the balance between competing activating and detoxifying reactions. While this balance is under genetic control, it is readily modulated by a variety of factors including nutritional status, age, hormones and exposure to drugs or other xenobiotics (Conney, 1982). If these factors, especially xenobiotics such as flavonoids, interact with these enzymes (Block and Gyllenhaal, 2002) and favour the activation reaction above the detoxification, it could result in reactive carcinogenic intermediate generation. In addition to this, herb-drug interactions can lead to toxicity, where the herb inhibits the metabolism of the drug, increasing the drug levels in the plasma. Conversely, it may increase the elimination process of the drug, rendering it ineffective. The latter may result in inability of the drug to suppress the tumour growth.

An increasing number of cancer patients are using complementary and alternative medicines (CAM) in combination with their conventional chemotherapeutic treatment. Therefore herb-drug interactions are becoming increasingly important. St John's wort (SJW) is very popular among cancer patients because of its supposed activity in depression (Mathijssen *et al*., 2002; Pal and Mitra, 2006). SJW has been found to interact with the anticancer drugs irinotecan (Mathijssen *et al*., 2002; Huang and Lesko, 2004) and imatinib (Frye *et al*., 2004).

Hyperforin, the active constituent of St. John's wort, binds to and activates the pregnane X receptor (PXR) (Figure 8). Upon activation, PXR forms a heterodimer with the 9-*cis* retinoic acid receptor (RXR), and this complex binds to the xenobiotic response elements (PXRE) in the cytochrome P450 3A4

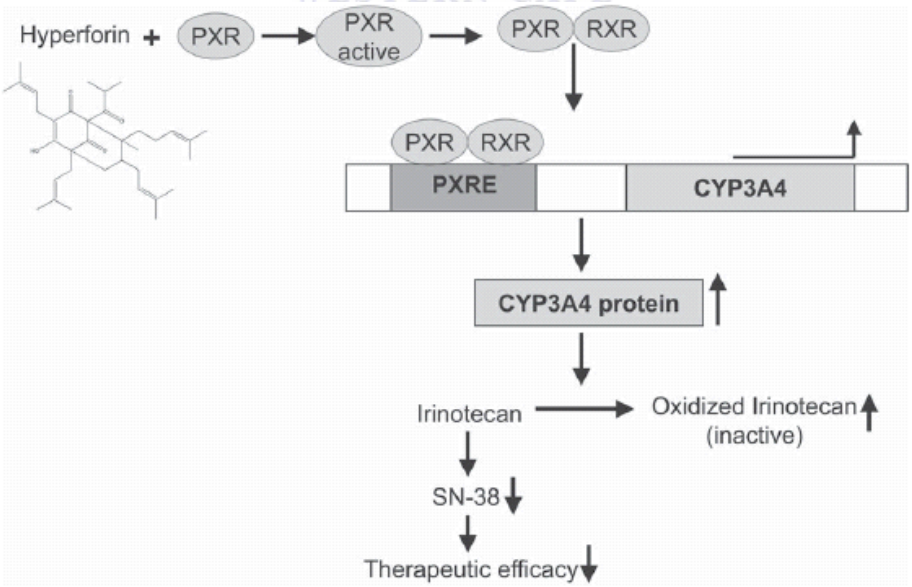


Figure 8 The effect of hyperforin on irinotecan therapy (Meijerman *et al*., 2006).

(*CYP3A4*) gene. The transcription of the gene is increased, and more CYP3A4 is formed, thereby increasing the metabolism of irinotecan into an inactive metabolite, SN-38 (oxidised irinotecan). The amount of irinotecan left to be metabolised into SN-38 decreases, leaving less active SN-38 and a lower therapeutic efficacy of irinotecan (Meijerman *et al*., 2006)

South African herbal teas

Tea has been consumed in China to promote health and longevity since 3000 BC. In South Africa only two Cape fynbos plants, *Aspalathus linearis*, better known as rooibos and various species of *Cyclopia*, commonly known as honeybush, have been successfully commercialised.

Aspalathus linearis (rooibos tea) is a member of the legume family of plants. It is only grown in a small area in the Cederberg region of the Western Cape Province. Rooibos was initially used by the Khoi-San for medicinal use before it was rediscovered by Dutch botanist Carl Thunberg in 1772 (Thunberg, 1795). Generally, the leaves are oxidized or fermented to produce the distinctive reddish-brown colour, but unfermented "green" rooibos is also produced. Rooibos flavonoids predominantly consist of dihydrochalcones, flavonols and flavones. Its flavonoid composition uniquely contains aspalathin (Rabe *et al*., 1994), nothofagin and a rare β-hydroxydihydrochalcone (Joubert, 1996). It has been found that fermentation of rooibos leaves decrease the aspalathin and nothofagin content (Joubert, 1996).

Cyclopia spp. (honeybush tea) is generally found on sandy soils of the coastal areas from Yzerfontein to the Cape peninsula. Honeybush is indigenous to the Cape of South Africa where it is used to make beverages and medicinal tea that has a pleasant, mildly sweet taste and aroma, somewhat like honey. The honeybush plant was noted in botanical literature by 1705. The bushes can grow up to 2m tall; however the leaf shape and size differ among the species, but most are thin, needle-like to elongated leaves. All the species are easily recognised in the field as they are covered with the distinctive, deep-yellow flowers, which have a characteristic sweet honey scent. Traditionally, the tea is harvested during flowering; either in early autumn or late spring, depending on the flowering period of the species. Three major phenolic constituents were identified in the leaves of *Cyclopia* species namely, mangiferin (a xanthone C-glycoside) and O-glycosides of flavonones hesperitin and isokuranetin (De Nysschen *et al*., 1996). However, Joubert *et al*., (2003) showed that the relative quantities of these phenolics vary between the different *Cyclopia* species and within a geographical area.

Both rooibos and honeybush herbal tea is caffeine-free and has very low tannin content (Greenish, 1881; Blommaert and Steenkamp, 1978; Terblanche, 1982; Morton, 1983; Yoshikawa *et al*., 1990; Hubbe and Joubert, 2000). Research has shown that rooibos and honeybush exhibit a vast array of health benefits. A study by Sasaki *et al*., (1993) in hamster ovary cells and male ICR mice showed that extracts of fermented rooibos tea decreased the number of chromosome aberrations and micronucleated reticulocytes, respectively, after exposure to

benzo(a)pyrene and mitomycin. Furthermore, fermented rooibos protected male Wistar rats against CCl_4–induced hepatic injury and showed histological regression of steatosis and cirrhosis in the liver tissue (Ulicna *et al*., 2003). Unfermented rooibos and honeybush (*C. intermedia*) also have a protective effect against methylbenzylnitrosamine-induced oesophageal papillomas in rats, decreasing it in size and number (Sissing, 2008). A study, conducted in the *Salmonella* mutagenicity assay, showed that hot water extracts of rooibos and honeybush exhibited protection against carcinogens requiring metabolic activation (Marnewick *et al*., 2000). Unfermented rooibos and honeybush significantly reduced the activation of aflatoxin B_1 and protected against 2-AAF-induced mutagenesis in cytosolic liver fractions of rats (Marnewick *et al*., 2004). Matsuda *et al*., (2007) showed that rooibos causes an increase in intestinal 1'- and 4-hydroxylation activities of CYP3A4 in rats as well as an increase in intestinal CYP3A4 content; although rooibos did not affect the concentration of microsomal liver CYP450. Both teas significantly increased the cytosolic glutathione-s-transferase α and microsomal UDP-glucuronosyl transferase activities, as well as the glutathione content (Marnewick *et al*., 2003), resulting in an increase in GSH/GSSG ratio.

CONCLUSIONS

Xenobiotic metabolising enzymes (cytochrome P450 and phase II and III) are responsible for activating or deactivating any foreign chemical an organism is exposed to. The fate of the xenobiotic depends on its chemical composition or

structure and its enzyme affinity. Some xenobiotics are metabolised by the same enzymes and thus compete for the same binding sites. Therefore, one xenobiotic can inhibit or enhance the metabolism of the other. This is known as herb-herb or herb-drug interactions. It is imperative to investigate these interactions, especially when studying the health properties of herbs. Herb-drug interactions can result in toxicity or ineffectiveness of pharmaceutical drugs. Many carcinogens are also metabolised by CYPs, which may enhance the formation of DNA-reactive metabolites, leading to an increased risk of tumor formation. The induction of certain phase II enzymes, however, can protect against the adverse effects by enhancing the deactivation and excretion of these DNA-reactive metabolites. Studies have shown that rooibos and honeybush herbal teas modulate hepatic phase II xenobiotic metabolising enzymes in rats, however, it has not previously been established whether the teas or their respective polyphenols affect xenobiotic metabolising enzymes at transcriptional level.

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CHAPTER 3

Effect of polyphenol-enriched herbal teas on the expression of genes encoding xenobiotic metabolising enzymes, in rat liver and kidneys

ABSTRACT

The use of polyphenol-enriched extracts by the nutraceutical and pharmaceutical industries has been controversial with respect to their potential health beneficial effects. At present very little information is available on the effect of polyphenol-enriched extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp.*) herbal teas on different cellular processes and their safety when used in dietary supplements. In the present study qPCR array analyses were used to investigate the effect of polyphenol-enriched extracts of rooibos and two honeybush species (*C. genistoides* and *C. subternata*) on the expression of hepatic and renal genes encoding the xenobiotic metabolising enzymes. Rats receiving the rooibos extract had the highest total polyphenol intake with the dihydrochalcone, aspalathin, as the major polyphenol. The major polyphenols consumed by the rats receiving the honeybush extracts were the xanthones, mangiferin and isomangiferin. The flavanone and flavone intake of the rats receiving the *C. subternata* extract was higher (P≤0.05) when compared to *C. genistoides* treated rats while the latter extract provided a higher xanthone intake.

In the liver both rooibos and honeybush up-regulated the genes encoding aldehyde dehydrogenase, glucose phosphate isomerase, while down-regulating the gene for 17β-hydroxysteroid dehydrogenase 2 (*Hsd17β2*). Cytochrome P450 (Cyp) 19a1 was selectively increased by rooibos while honeybush (*C. genistoides*) up-regulated alcohol dehydrogenase and down-regulated *Hsd17β1*. The effect of *C. subternata* was different, up-regulating carboxyl esterases 1 and 2, Cyp2e1, while down-regulating

Cyp's 2b6 and 2c13. In the kidneys the genes encoding the phase II enzymes, glutathione–S-transferase (*Gstm1*) and microsomal *Gst1*, were up-regulated by rooibos, whereas the ATP binding cassette transporter (*Abcb1*), *Cyp4b1*, gamma glutamyltransferase 1 (*Ggt1*) and N-acetyltransferase 1 (*Nat1*) were down-regulated. *Cyclopia genistoides* up-regulated the *Cyp19a1*, *Cyp1a1* and *Hsd17β3* encoded genes, but down-regulated the genes of *Gstm3* and 5 and hexokinase 2 (*Hk2*). *Cyclopia subternata*, on the other hand, up-regulated the genes encoding arachidonate 15-lipoxygenase (*Alox15*), aryl hydrocarbon receptor nuclear translocator (*Arnt*), *Cyp1b1*, fatty acid amide hydrolase (*Faah*), *Gstm1* and 4, *Mgst2* and myeloperoxidase (*Mpo*), but down-regulated the esterase 22 (*Es22*), *Gstm5* and *Hk2* encoded genes. The results indicated that differences exist in the modulation of xenobiotic metabolism at the transcriptional level, depending on the tissue type and the herbal tea utilised, presumably related to specific polyphenol constituents.

Keywords: *Aspalathus linearis*, *Cyclopia spp*, xenobiotic metabolising enzymes, qPCR array.

INTRODUCTION

Xenobiotics are foreign substances entering the body and can either be beneficial or harmful depending on the dose and/or metabolic fate. Examples of xenobiotics include environmental carcinogens such as the polyaromatic hydrocarbons (PAH) and tobacco-related nitrosamines and pharmaceutical drugs, as well as a vast array of naturally occurring plant polyphenols. These xenobiotics may interact with the cell's metabolic pathways and basic cellular constituents such as RNA, DNA and proteins (Boyland and Green, 1962), often leading to the modulation of normal cellular functions.

Upon entering an organism most xenobiotics undergo metabolism that is catalysed by the xenobiotic metabolising enzymes (XMEs) which play a central role in the detoxification and/or elimination of the chemicals from the body (Meyer, 1996). These cellular processes include the phase I and phase II metabolising enzymes and phase III transporters, which are present in abundance either at the basal and/or at induced levels after exposure to xenobiotics (Meyer, 1996; Rushmore and Kong, 2002; Wang and LeCluyse, 2003). In general, it appears that exposure to xenobiotics and the interaction with metabolising enzymes (phase I and phase II) and phase III transporters may trigger cellular "stress" responses leading to an increase in gene expression (Plant, 2007; Wahli and Martinez, 1991), which ultimately enhances the elimination and clearance of xenobiotics (Xu *et al*., 2005). Consequently, these homeostatic responses play a central role in the protection of cells against "environmental" insults such as those elicited by exposure to xenobiotics.

Indigenous to South Africa, the herbal teas *Aspalathus linearis* (rooibos herbal tea) and *Cyclopia* spp. (honeybush herbal tea) have become increasingly popular for their unique flavour, as well as anecdotal health properties. Traditional medicinal uses of rooibos in South Africa include alleviation of infantile colic, allergies, asthma and dermatological problems, while a decoction of honeybush was used as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis (Bowie, 1830; Morton, 1983; Rood, 1994; Joubert *et al*., 2008). Since their global commercialisation, researchers have been investigating the health properties of the herbal teas and postulate their protective role against several human diseases. A study has shown that fermented rooibos herbal tea protected male Wistar rats against carbon tetrachloride (CCl_4)–induced hepatic injury and caused histological regression of steatosis and cirrhosis in the liver tissue (Ulicna *et al*., 2003). Both herbal teas also significantly reduced the metabolic activation of aflatoxin B_1 and protected against 2-acetylaminofluorene (2-AAF)-induced mutagenesis (Marnewick *et al*., 2000 and 2004). Unfermented rooibos and honeybush herbal teas enhanced the activity of glutathione-S-transferase and UDP-glucuronosyltransferase in rat liver (Marnewick *et al*., 2003).

Although rooibos and honeybush herbal teas exhibit beneficial health properties, it is not known whether extracts of these herbal teas, in particular enriched in polyphenol content could modify the expression of the xenobiotic metabolising enzymes. In the present study polyphenol-enriched rooibos and honeybush herbal tea extracts were investigated for their *in vivo* effect on the expression of genes encoding xenobiotic metabolising enzymes in rat liver and kidney. The extracts were used to further

elucidate their potential beneficial and/or adverse effects when used as antioxidant supplements.

MATERIALS AND METHODS

Chemicals

Analytical grade methanol, ethanol, hydrochloric acid, and sodium carbonate (Na_2CO_3) were purchased from Merck Chemicals (Germiston, South Africa). Gallic acid (GA), dimethyl-sulfoxide (DMSO), and Folin-Ciocalteau (F-C) reagent were obtained from Sigma-Aldrich (South Africa) as was the molecular biology grade β-mercaptoethanol (β-ME). Orientin and iso-orientin were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), mangiferin and hesperidin from Sigma-Aldrich (St Louis, MO, USA) and vitexin, isovitexin, hyperoside, luteolin, chrysoeriol, rutin, narirutin and hesperetin from Extrasynthese (Genay, France). The HPLC Far UV gradient grade acetonitrile was obtained from BDH (VWR International, Poole, UK) and acetic acid from Riedel-de Haën (Seelze, Germany). Aspalathin and nothofagin were purified from unfermented rooibos at the PROMEC Unit of the Medical Research Council, Tygerberg, South Africa to a purity of >95%.

Chemical analysis of herbal tea samples

The polyphenol-enriched herbal tea extracts (*Aspalathus linearis*, *Cyclopia genistoides* and *C. subternata*) were supplied by the RAPS Foundation (Germany). The preparation was according to US patent application US2008/0247974 A1.

The total polyphenol (TP) content of the enriched extracts was determined by using the scaled-down Folin-Ciocalteau (F-C) method with gallic acid as standard. Six gallic acid standard solutions, ranging from 50-500 µl, and extract stock solutions, prepared in distilled water, were diluted in triplicate in distilled water to a final volume of 5 ml. F-C reagent (10%) (5 ml) and 7.5% Na_2CO_3 (4 ml) were then added to either a 1 ml standard, extract solution or distilled water as a blank, to a final volume of 10 ml, vortex mixed and incubated for 2 hrs at 37°C. The absorbance was determined at 765 nm and the TP content expressed as gram (g) gallic acid equivalents (GAE) per 100 g extract.

Analyses of monomeric polyphenols by high performance liquid chromatography (HPLC)

An Agilent 1200 HPLC system consisting of a quaternary pump, autosampler, in-line degasser, column oven and diode-array detector (Agilent Technologies Inc., Santa Clara, USA) with Chemstation 3D LC software was used for HPLC-DAD analysis. Stock solutions of all standards were prepared in DMSO and aliquots frozen at -20°C. Extracts were dissolved in deionised water (ca 6 mg/mL). Standard calibration mixtures were prepared in a range of concentrations by dilution in HPLC grade water. Ascorbic acid was added to the standard calibration mixtures and samples (final concentration ca 10 mg/mL) before filtering with 0.45 µm pore-size Millex-HV syringe filter devices (Millipore) prior to HPLC analysis. UV-Vis spectra from 220 to 400 nm were recorded for all analyses. Peaks were identified by comparing retention times and UV-Vis spectra with those of authentic standards.

Rooibos herbal tea: Separation, according to a modified version of the method by Joubert (1996), was achieved on a Gemini C18 (150 × 4.6 mm, 5 µm particle size, 110 Å pore size; Phenomenex, Torrance, USA) column protected by a guard column with the same stationary phase at 38°C and a flow rate of 0.4 mL/min. Solvent A, 2% acetic acid (v/v), and B, methanol, were used in the following solvent gradient: 0–3 min (20% B); 15 min (30% B); 18 min (30% B); 27 min (35% B); 29 min (35% B); 35 min (40% B); 54 min (60% B); 62 min (80% B); 68 min (60% B); 81 min (20% B); 90 min (20% B at 1.2 mL/min). Aspalathin and nothofagin were quantified at 288 nm, while 350 nm was used for quantification of the other compounds.

Honeybush herbal teas: Separation was carried out according to the method of De Beer and Joubert (2010) on a Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5 µm particle size, 80 Å pore size; Agilent Technologies) column protected by a guard column with the same stationary phase at 30°C. Solvent A, 0.1% formic acid (v/v), and B, acetonitrile, were used in the following solvent gradient at a flow rate of 1 mL/min: 0–6 min (12% B); 7 min (18% B); 14 min (25% B); 19 min (40% B); 24 min (50% B); 29 min (12% B); and 40 min (12% B). The xanthones were quantified at 320 nm, the flavanones at 288 nm and the flavones at 350 nm. Scolymoside and the eriodictyol-glycoside were quantified as luteolin and hesperidin equivalents, respectively.

Animals, treatment and clinical biochemical parameters

The animal study was conducted by Ms J.D. van der Merwe of the Department of Food Science, University of Stellenbosch as part of her PhD degree. The use of

experimental animals was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council (MRC) (Letter attached in Addendum II).

Seven to eight weeks old male Fischer rats (n=10) weighing between 150-200 g were obtained from the Primate Unit of the Medical Research Council (Tygerberg, South Africa). The animals were housed individually in stainless steel wire-bottomed cages fitted with Perspex houses in a room illuminated for 12 hr photoperiods at 50% humidity and a constant room temperature of 24-25℃. Freeze-dried extracts of aspalathin-enriched green rooibos (18% aspalathin), mangiferin-enriched green *C. subternata* (3.8% mangiferin) and *C. genistoides* (9.3% mangiferin) were mixed into mash, prepared from feed cubes (Epol Ltd, Cape Town, South Africa), by milling, respectively, using a food mixer. Rats had free access to food and water.

The different herbal tea diets were fed for 28 days, whereas the control rats (n=10) received only the feed. The feed intake was monitored every second day while the body weight was recorded once weekly (Tables 1 and 2, Addendum II). Following the 28 day feeding period, rats were fasted overnight and terminated by cervical dislocation. Blood was collected in SST sterile tubes (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK) and serum prepared by centrifugation at 2,000 x*g* for 10 minutes at 4 ºC.

Serum clinical biochemical parameters were determined on a Technikon RA 1000 automated analyzer (NIRS, MRC of South Africa) and included creatinine, alkaline phosphatase (ALP), total cholesterol, total iron, aspartate transaminase (AST), alanine transaminase (ALT) and total (TBili) and unconjugated (DBili) bilirubin. The liver and

kidney tissues were harvested and weighed, and sections (200 – 400 mg) of each tissue type were immediately snap frozen in liquid nitrogen and stored at -80℃ for investigating changes in the gene expression patterns.

Gene expression analyses

Extraction of ribonucleic acid (RNA)

Total RNA was isolated from the liver and kidney tissue of rats in each group using the RNeasy mini kit* (Qiagen, Valencia, CA, USA). This technology combines the selective binding properties of a silica-gel-based membrane with the speed of micro-spin technology to extract RNA in the presence of a specialised high-salt buffer system. In short, liver and kidneys tissue (20-25 mg) were lysed in 600 µl of a highly denaturing guanidine isothiocyanate (GITC)-containing lyses buffer (RLT) and homogenised in a Tissuelyser II (Qiagen, Valencia, CA, USA) for 40 sec.

Beta-mercaptoethanol (β-ME) (14.3 M) was added to the RLT buffer (10 µl/ml) prior to use. The lysate was centrifuged at 10,000 ×*g* for 3 min and the supernatant transferred to a clean micro-centrifuge tube. Ethanol (75%; 600 µl) was added and the mixture fractionated on an RNeasy spin column by centrifugation at 8,000 ×*g* for 2x15 sec. The RNeasy spin column membrane was washed with RW1 buffer (700 µl) by centrifugation at 8,000 ×*g* for 15 sec. A final wash step, using RPE buffer (500 µl), was performed by a two-step centrifugation, first for 15 sec and then again for 2 min at 8,000 ×*g*. The RNA was eluted from the spin column membrane with RNase-free water (50 µl) and centrifugation at 8,000 ×*g* for 1 min.

**The description, concentrations and pH of the buffers are not defined in the RNA extraction kit user manual.*

RNA cleanup and quality assessment

Genomic DNA in the RNA samples was digested using the Ambion® Turbo DNA-free™ kit (Applied Biosystems, South Africa) according to the manufacturer's recommendations. Once again the buffer strength and pH were not available. Briefly, RNA (20 µg), DNase buffer, DNAse I (4 units) and nuclease-free water were mixed and incubated for 90 min at 37℃ in a final volume of 20 µl. The DNAse enzyme was then deactivated by incubating the mixture with 10 µl DNAse inactivation reagent for 2 min at room temperature. In order to collect the RNA-containing supernatant, the mixture was centrifuged at 8,000 ×*g* for 15 min.

The RNA was quantified spectrophometrically by placing 2 µl RNA on the pedestal of a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) (Table 4, Addendum II) and the RNA quality was analysed on an Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbron, Germany). The Agilent Bioanalyzer provides a platform that uses a fluorescent assay involving electrophoretic separation to evaluate RNA samples qualitatively. The software creates an electrogram, which diagrams the fluorescence over time. Briefly, a gel-dye mixture (9 µl) was transferred to a RNA 6000 Nano Labchip and spread across by plunging 1 ml of air, using a syringe, into the chip for 30 sec. The gel-dye mix (9 µl) was also transferred to two additional wells for quality control purposes. A Nano marker buffer was added to all sample wells and the RNA ladder well. The RNA samples (400 ng in 1 µl) and RNA 6000 ladder (1 µl) (Ambion)

were denatured at 70°C for 2 min before loading into their respective wells. The chip was vortexed at 240 rpm for 1 min, developed and analysed on the Bioanalyzer using an Agilent software program. The small (18S) and large (28S) ribosomal RNA (rRNA) subunits were recorded as bands and peaks on an electrogram. RINs (RNA integrity numbers), displayed as numbers from 1 to 10, were determined from the ratio between the size of the 28S band to the 18S band.

Following the RNA quality assessment, the RNA samples were stored at -80°C prior to gene analyses. Of the 10 liver and kidney samples available for each treatment group, three samples were selected for qPCR array analyses while four liver samples per treatment group were selected for the gene validation assay. The sample selection was based on the best RNA integrity parameters (Figures 1 and 2, Addendum II).

Reverse transcription (RT)

First strand complimentary deoxyribonucleic acid (cDNA) synthesis was carried out using the isolated RNA (three samples per group) and RT^2 First Strand Kit (SA Biosciences, Frederick, Maryland, USA) according to the manufacturer's protocol. Briefly, contaminating genomic DNA was eliminated from total RNA (1 µg) with a five times concentrated genomic DNA elimination buffer (2 µl) and nuclease-free water to a final volume of 10 µl and incubated at 42°C for 5 min. The sample was subsequently mixed with oligo-dT primers, a five times concentrated reverse transcription buffer and reverse transcriptase to a final volume of 20 µl and incubated 42°C for 15 min. The RT reaction was terminated by heating at 95°C for 5 min and stored at -20°C until use.

qPCR array analyses in the liver and kidneys

The resultant cDNA (20 µl) was further diluted in nuclease-free water to a final volume of 111 µl. A subsample (102 µl) was mixed with a twice concentrated RT^2 qPCR SYBR green I master mix (550 µl) (SA Biosciences), containing HotStart DNA polymerase, and nuclease-free water (448 µl) to a final volume of 1100 µl. The cDNA mixture (10 µl) was transferred to a polymerase chain reaction (PCR) profiler array (SA Biosciences) containing 84 genes, coded for xenobiotic metabolising enzymes and transporters, five reference genes and quality control parameters (Table 3, Addendum II). Data were generated in real time from a ABI 7900HT real time PCR apparatus (Applied Biosystems, South Africa) with a two-step cycling program (1 cycle at 95°C for 10 min; 40 cycles at 95°C for 15 sec and then at 60°C for 1 min). All samples were analysed in triplicate. The data were analysed on the Microsoft Excel-based PCR array data analysis template from the SA Biosciences website. (http://www.superarray.com/pcrarraydataanalysis.php)

Genomic DNA and reverse transcription controls

The cycle threshold (Ct) reflects the cycle number at which the fluorescence generated within a reaction exceeds the background noise and this inversely correlates to the initial amount of target template. The mean Ct for each sample's genomic DNA control (GDC) parameter was determined (Table 5, Addendum II). A value greater than 35 indicates that the genomic DNA contamination is too low to affect the gene expression profiling results. For the samples that were between 30 and 35, a gene-by-gene analysis for each gene of interest (GOI) was done using the equation $\Delta Ct_{(GOI)}$ = Ct

(GDC) – Ct (GOI). If the value is six or greater, the results may still be used without further validation. However, if the value is less than six, the results should be validated with individual gene-specific primer assays that include a no reverse transcription (NRT) control.

The reverse transcription control (RTC) indicates whether the concentrations of impurities in the RNA sample are enough to inhibit the reverse transcription reaction. RTC signifies the difference between the average Ct of the RTC and the positive PCR control (PPC). A value below 5 is evidence of no RT inhibition.

Data normalisation and analysis

Five reference (housekeeping) genes [ribosomal protein large P1 (*Rplp1*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), ribosomal protein L13A (*Rpl13a*), lactate dehydrogenase A (*Ldha*) and β-actin (*Actb*)] were used for normalisation. The mean Ct of each cDNA sample for each target gene and all reference genes were determined. $\Delta Ct_{(GOI)}$ is the difference in Ct between the target gene and reference gene. For example, if the target gene (*Cyp1a1*) has an average Ct value of 25 and the average Ct value of the reference gene 10, then the $\Delta Ct_{(Cyp1a1)}$ will be equal to 15.

The comparative Ct (ΔΔCt) method was used to calculate the relative amount of transcripts in the treated and untreated samples (control): ΔΔCt = ΔCt (treated) – ΔCt (control). The fold-change for each treated sample relative to the control sample was calculated using the formula $2^{-\Delta\Delta Ct}$. Statistical calculations were done based on the ΔCt

values using a two-tailed t-test. A P-value equal to and/or less than 0.05 was designated as significant.

Validation of selected genes in the liver using qPCR

Current publication guidelines require that all microarray results be confirmed by an independent gene expression profiling method. Although it is not necessary for PCR arrays (Gaj *et al*., 2008), quantitative real time PCR was used to validate the PCR array results in this study to confirm that the results are accurate. The first step in confirming array results by real-time PCR is the selection of gene-specific primer pairs. The following were selected from the list of genes that were analysed on the microarrays: Aldehyde dehydrogenase 1a1 (*Ald1a1*), because it was affected by all three extracts, catechol-O-methyltransferase (*Comt*), since its enzyme is known to be associated with polyphenol metabolism, cytochrome P450 1a1 (*Cyp1a1*), is one of the major CYPs, cytochrome P450 2e1 (*Cyp2e1*) had a fold difference of less than 1.5, 17β- hydroxysteroid dehydrogenase 2 (*Hsd17β2*) was affected by all extracts, sulfotransferase 1e1 (*Sult1e1*) because of its known association with polyphenol metabolism and *Hprt1* as a reference gene for data normalisation (Table 1).

Reverse transcription

Four RNA samples per group were used for the validation of selected genes. Total RNA (10 µg), in a volume of 10 µl, was reversed transcribed into cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, South Africa) according to the manufacturer's protocol. Briefly, RNA was mixed with 25 times concentrated

deoxyribonucleotide triphosphate (dNTP) mix (100 mM), 10 times concentrated RT Random primers, MultiScribe reverse transcriptase, 10 times concentrated reverse transcription buffer and nuclease-free water to a final volume of 20 µl. The thermal cycler (Eppendorf, Germiston, South Arica) conditions were as follows: 25℃ for 10 min, 37℃ for 120 min and 85℃ for 5 sec. The cDNA was then stored at -20℃ until further use.

Primer design and optimisation

Gene-specific qPCR primers (Table 1) were designed using the Primer Express® software v2.5 (Applied Biosystems, South Africa) and synthesised by Integrated DNA technologies (IDT, Whitehead Scientific, South Africa).

Table 1 Primer sequences of selected genes for qPCR analyses.

Gene	***Genebank***	***Sense***	***Antisense***
		5'→3'	***5'→3'***
Ald1a1	NM_022407	CAAGGCAGATGTTGACAAAGCT	GGGAGCCAATCTGGAAAGC
Comt	NM_012531	CCATGGAGATGAACCCTGACTAC	TAGGCCTGCAAAGTTCAGCATT
Cyp1a1	NM_012540	GCCCCGGCTTTCTGACA	CGGAAGGTCTCCAGGATGAAG
Cyp2e1	NM_031543	TTGGCCGACCTGTTCTTTG	TTTCTGGGTATTTCATGAGGATCA
Hprt1	NM_012583	TCAAGCAGTACAGCCCCAAAAT	CAACACTTCGAGAGGTCCTTTTC
Hsd17β2	NM_024391	CGCAGAGAAGATACAAGACAAAGG	AAAGTGCAAGACCCCAGCAT
Sult1e1	NM_012883	TCCAGGAGATGAAGAACAATCCA	AGGGCTTCAGGGAAGTGGTT

Aldehyde dehydrogenase 1a1 (*Ald1a1*), *catechol-O-methyltransferase* (*Comt*), *cytochrome P450 1a1* (*Cyp1a1*), *cytochrome P450 2e1* (*Cyp2e1*), *17β- hydroxysteroid dehydrogenase 2* (*Hsd17β2*), *sulfotransferase 1e1* (*Sult1e1*) and *hypoxanthine guanine phosphoribosyl transferase 1* (*Hprt1*).

Quantitative real-time PCR (qPCR) of liver samples

Real-time PCR was carried out in duplicate in a 25 µl amplification mixture containing 2 µl of template cDNA, 12.5 µl of two times concentrated SYBR Green I Master Mix (Applied Biosystems, South Africa), and 1.25 µl each of 22.5 µM sense and antisense primers. The PCR conditions included a polymerase activation step at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec and developed on an ABI Real-time PCR 7500 system (Applied Biosystems, South Africa). A non-template control was included for all of the primer pairs to eliminate the possibility of significant DNA contamination. Messenger RNA (mRNA) levels from each treated and untreated rat were normalised to the corresponding reference gene, *Hprt*'s mRNA levels. The gene expression levels from the control were set as 1 to compare the relative amounts of gene expression levels in the experimental groups. Statistical analysis (Anova) of the results was performed in Microsoft Excel. Significant difference was determined with $P \leq 0.05$ by the Student *t* test.

RESULTS

Analyses of monomeric polyphenols by HPLC

The dihydrochalcone *C*-glucoside, aspalathin, was found to be the most abundant flavonoid in the rooibos extract followed by the flavonols, rutin+isoquercitrin+quercetin-3-robinobioside, the dihydrochalcone, nothofagin and the flavone oxidation products of aspalathin, isoorientin and orientin (Table 2). Only small amounts of vitexin, isovitexin, hyperoside, luteolin-7-glucoside, luteolin and chrysoeriol were recorded, as well as traces of quercetin.

Of the honeybush polyphenolic constituents, the xanthones, mangiferin and isomangiferin, were found to be the most abundant. *Cyclopia genistoides* had a higher xanthone content, whereas *C. subternata* had either similar or higher flavanone and flavone levels. Of interest is the high eriocitrin content of *C. subternata* and the presence of scolymoside. The chemical structures of these polyphenols are presented in Figure 1.

Feed, herbal tea extract and polyphenol intake

The feed intake of the rats between the different dietary treatments and control was not significantly different (Table 3). Rats receiving the rooibos extract, containing almost twice the total polyphenol content of the honeybush extracts, have the highest total polyphenol intake followed by *C. subternata* with *C. genistoides* having the lowest intake.

Table 2 Monomeric rooibos and honeybush polyphenolic constituents of the enriched extracts incorporated in the rat diets.

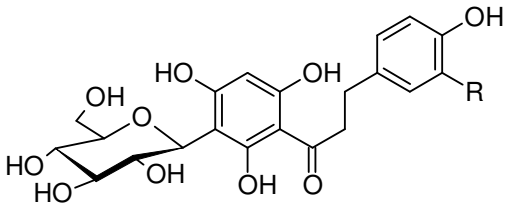
Aspalathus linearis (Rooibos)

Polyphenol subgroup	***Compound***	***g/100g Soluble solids (SS)***
Dihydrochalcone	Aspalathin	18.73
	Nothofagin	1.45
Flavone *C*-glycoside	Isoorientin	1.45
	Orientin	0.81
	Vitexin	0.30
	Isovitexin	0.39
Flavonol	Rutin+Isoquercitrin+Quercetin-3-robinobioside	2.10
	Quercetin	Traces
	Hyperoside	0.29
Flavone	Luteolin	0.06
	Chrysoeriol	0.02
	Luteolin-7-glucoside	0.04

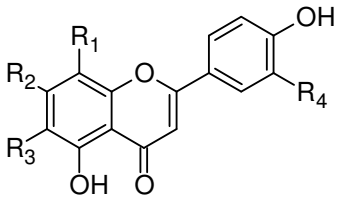
Cyclopia spp. (Honeybush)

Polyphenol subgroup	***Compound***	***C. genistoides g/100g SS***	***C. subternata g/100g SS***
Xanthones	Mangiferin	9.29	3.79
	Isomangiferin	3.29	1.30
Flavanones	Hesperidin	0.83	0.99
	Eriocitrin	nd	1.1
	Naringenin	0.01	0.01
	Eriodictyol-glycoside	0.03	0.23
Flavone	Luteolin	0.09	0.06
	Scolymoside	nd	1.29

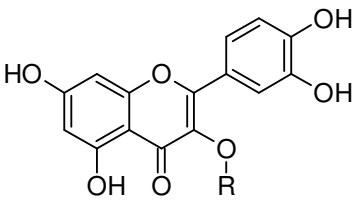
HPLC analysis was done by Dr D de Beer, ARC Infruitec-Nietvoorbij, Stellenbosch.
All values are expressed as a percentage of the soluble solids (g/100g SS).
Values are means of duplicate analysis.
nd- not detected.
Rutin, isoquercrtrin and quercetin-3-robinobioside co-eluted under the HPLC separation conditions.



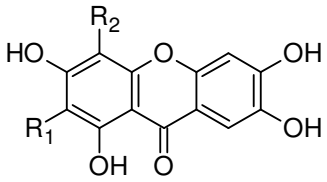
Dihydrochalcones
aspalathin: R = OH
nothofagin: R = H



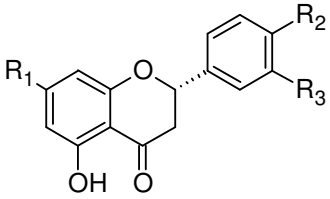
Flavones
orientin: R_1 = *C*-β-D- glucosyl, R_2 = R_4 =OH, R_3 = H
iso-orientin: R_1 = H, R_2 = R_4 =OH, R_3 = *C*-β-D-glucosyl
vitexin: R_1 = *C*-β-D-glucosyl, R_2 = OH, R_3 = R_4 = H
isovitexin: R_1 = R_4 = H, R_2 = OH, R_3 = *C*-β-D-glucosyl
luteolin: R_1 = R_3 = H, R_2 = R_4 = OH
chrysoeriol: R_1 = R_3 = H, R_2 = OH, R_4 = OCH_3
scolymoside: R_1 = R_3 = H, R_2 = *O*-β-D-rutinosyl, R_4 = OH



Flavonols
quercetin: R = H
rutin: R = *O*-β-D-rutinosyl
isoquercitrin: R = *O*-β-D-glucosyl



Xanthones
mangiferin: R_1 = *C*-glucosyl; R_2 = H
mangiferin: R_1 = H; R_2 = *C*-glucosy;



Flavanones
hesperidin: R_1 = *O*-β-D-rutinosyl; R_2 = CH_3; R_3 = OH
eriocitrin: R_1 = *O*-β-D-rutinosyl; R_2 = H; R_3 = OH
naringenin: R_1, R_2 = OH; R_3 = H

Figure 1 Chemical structures of the polyphenolic constituents of A. linearis, C. genistoides and C. subternata.

Table 3 Feed intake of the rats and a representation of the extract and total polyphenol intake per 100 g body weight.

Treatment	*TP (g gallic acid/ 100 g extract)*	*Feed intake (g feed/100 g bw)*	*Extract intake (g extract/100 g bw)*	*TP intake (mg GAE/ 100 g bw)*
Control	-	9.73 ± 0.42a	-	-
A. linearis	39.22 ± 1.10a	9.65 ± 0.39a	0.19 ± 0.01a	75.66 ± 3.04a
C. genistoides	21.88 ± 0.59c	9.97 ± 0.49a	0.25 ± 0.01b	54.55 ± 2.70b
C. subternata	24.83 ± 0.82b	10.35 ± 0.45a	0.26 ± 0.01b	64.27 ± 2.77c

Data form part of J.D. van der Merwe PhD thesis.
Values are means ± standard deviation (n = 10), except for C. genistoides (n = 9).
Rooibos and honeybush enriched extracts were incorporated at a 2 and 2.5% level in the diets, respectively.
TP- Total polyphenols. GAE – Gallic acid equivalents, bw - body weight.

Individual polyphenol intake

In the rooibos group, intake of aspalathin was the highest (Table 4), followed by the flavonols. Equal levels of nothofagin and isoorientin were ingested followed by orientin. The intake of the nothofagin oxidation products, vitexin and isovitexin, and the flavonol, hyperoside, was similar, followed by the minor flavones, the aglycones, chrysoeriol and luteolin. The rats that received the *C. genistoides* extract ingested more than twice the amount of the xanthones, mangiferin and isomangiferin, compared to the group that received the *C. subternata* extracts. However, the intakes of the flavanones, eriocitrin, hesperidin and eriodictyol-glycoside, and the flavone scolymoside, were significantly (P≤0.05) higher in the *C. subternata* treated rats, whereas the *C. genistoides* treated rats had a higher (P≤0.05) luteolin intake.

Table 4 Daily rat intakes of individual rooibos and honeybush monomeric polyphenols.

Polyphenols	***A. linearis*** *(mg per 100 g rat BW)*
Aspalathin	36.13 ± 1.45
Nothofagin	2.8 ± 0.11
Orientin	1.56 ± 0.06
Isoorientin	2.8 ± 0.11
Vitexin	0.58 ± 0.02
Isovitexin	0.75 ± 0.03
Rutin/isoquercitrin	4.05 ± 0.23
Luteolin	0.12 ± 0.01
Chrysoeriol	0.39 ± 0.02
Hyperoside	0.56 ± 0.02

Honeybush	***C. genistoides*** *(mg per 100 g rat BW)*	***C. subternata*** *(mg per 100 g rat BW*
Mangiferin	23.16 ± 1.15a	9.81 ± 0.42b
Isomangiferin	8.20 ± 0,41a	3.37 ± 0.15b
Hesperidin	2.07 ± 0.10a	2.56 ± 0.11b
Luteolin	0.22 ± 0.01a	0.12 ± 0.01b
Eriocitrin	-	2.85 ± 0.12
Naringenin	0.02 ± 0.001a	0.03 ± 0.001a
Eriodictyol-glycoside	0.07 ± 0.004a	0.60 ± 0.03b
Scolymoside	-	3.34 ± 0.14

Data form part of Debora van der Merwe's PhD thesis.
Means followed by the same letter do not differ significantly, if letter differs then P≤0.05.
Values represent the average daily polyphenol intake (n=10).

Body weight and serum clinical biochemistry parameters

There were no significant changes in the body weight gain and relative liver and kidney weights between the different treatment and control rats (Table 5). Of the serum clinical biochemical parameters only the total bilirubin was significantly increased (P≤0.05) by *C. subternata*, while a marginal (P<0.1) effect was noticed for *C. genistoides*. *Cyclopia subternata* significantly (P≤0.05) reduced the level of alanine aminotransferase (AST), while a marginal reduction was noticed for *C. genistoides*.

Gene expression analyses

qPCR array analysis of xenobiotic metabolising enzymes in the liver

Of the 84 genes analysed, only 11 were significantly (P≤0.05) affected in the liver (Table 6). The genes, *Ald1a1*, *Cyp19a1* and glucose phosphate isomerase (*Gpi*), were up-regulated by the polyphenol-enriched rooibos herbal extract, whereas *Hsd17β2* was down-regulated. *Cyclopia genistoides* up-regulated the genes of alcohol dehydrogenase (*Adh4*), *Ald1a1* and *Gpi*, however, it down-regulated the genes of both *Hsd17β1* and *Hsd17β2. Cyclopia subternata* up-regulated the genes *Ald1a1*, carboxyl esterases 1 and 2 (*Ces1* and *2*), *Cyp2e1* and *Gpi*, but down-regulated *Cyp2b6*, *Cyp2c13* and *Hsd17β2*.

The major CYPs, *Cyp3A* and *1a1/1a2*, nuclear receptors, ABC transporters and phase II transferases, glutathione-S-transferase (*Gst*), sulfotransferase (*Sult*), UDP-glucuronyltransferase (*Ugt*) and N-acetyltransferase (*Nat*) were not significantly affected by the extracts (Table 6, Addendum II).

Table 5 Body weight gain, relative liver and kidney weight, and serum clinical biochemical parameters of rats fed the polyphenol-enriched herbal tea extracts for 28 days.

Treatment	BW gain (g)	Relative liver weight (%/bw)	Relative kidney weight (%/bw)	Chol (mmol/ L)	Iron (µmol/L)	ALP (U/L)	ALT (U/L)	AST (µmol/L)	TBili (µmol/L)	DBili (µmol/ L)	Creat (µmol/L)
Control	94.50a (10.20)	3.57a (0.18)	0.65a (0.03)	1.17a (0.18)	11.04a (1.06)	126.80a (11.36)	44.20a (9.90)	104.90a (21.93)a	8.26a (1.75)	0.99a (0.41)	46.19a (4.54)
A. linearis	89.50a (12.94)	3.70a (0.18)	0.63a (0.03)	1.15 a (0.09)	11.34a (1.27)	136.90a (6.43)	42.40a (5.04)	105.40a (12.96)a	7.78a (1.21)	0.84 a (0.21)	46.09a (2.72)
C. genistoides	92.00a (9.70)	3.81a (0.25)	0.67a (0.03)	1.18 a (0.07)	11.74a (2.12)	135.11a (10.92)	43.67a (5.87)	89.33ab (6.73)ab	8.60ab (2.55	1.05a (0.57)	45.34a (1.22)
C. subternata	93.50a (12.83)	3.57a (0.19)	0.66a (0.03)	1.19a (0.13)	11.41a (1.30)	128.00a (7.31)	41.90a (4.41)	88.30bc (5.10)	10.51bc (1.38)	1.24a (0.33)	45.66a (2.92)

Data form part of J.D. van der Merwe's PhD thesis.
Values are means ± STD (in parenthesis) of n = 10 rats (with the exception of C. genistoides, n = 9).
Means in a column followed by the same letter are not significantly different P≥0.05).
Abbr: Cholesterol (Chol), Alkaline phosphate (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Total bilirubin (TBili), Direct/unconjugated bilirubin (DBili), Creatine (Creat), body weight (BW).

Table 6 qPCR array analysis of liver tissue after exposure to polyphenol-enriched herbal teas for 28 days.

Gene	*A. linearis*		*C. genistoides*		*C. subternata*	
	Fold difference	P-value	Fold difference	P-value	Fold difference	P-value
Adh4			1.33	0.0429		
Ald1a1	2.39	0.0488	1.672	0.0283	2.47	0.0278
Ces1					1.32	0.0396
Ces2					1.61	0.0016
Cyp19a1	1.98	0.0272				
Cyp2b6					-1.29	0.0332
Cyp2c13					-1.33	0.0349
Cyp2e1					1.23	0.0345
Gpi	2.48	0.0068	2.17	0.0053	1.92	0.0131
Hsd17β1			-1.72	0.041		
Hsd17β2	-2.42	0.0078	-3.41	0.0138	-2.09	0.0421

P≤0.05 designated as significant.
- indicates down-regulation; + indicates up-regulation.
Alcohol dehydrogenase 4 (Adh4), aldehyde dehydrogenase 1a1 (*Ald1a1*), carboxyl esterase (Ces1&2), cytochrome P450 19a1 (Cyp19a1), cytochrome P450 2b6 (Cyp2b6), cytochrome P450 2c13 (Cyp2c13), *cytochrome P450 2e1* (*Cyp2e1*), glucose phosphate isomerase (Gpi), *17β- hydroxysteroid dehydrogenase 1&2* (*Hsd17β1&2*).

Quantitative real-time PCR (qPCR) of liver samples

The mRNA of the selected genes was normalised against the reference gene *Hprt1*. Compared to the control, rooibos showed the same level of *Cyp2e1* mRNA, whereas *C. genistoides* and *C. subternata* slightly decreased its quantity. Whilst no significant change in mRNA quantity was observed for *Comt*, all three herbal tea extracts decreased *Hsd17β2* transcription.

Some inconsistencies were observed in the qPCR results, which could be due to poor primer design or very little mRNA present in the liver tissue for *Ald1a1*, C*yp1a1* and *Sult1e1 and* were therefore omitted.

qPCR array analysis of xenobiotic metabolising enzymes in the kidneys

Of the 84 xenobiotic metabolism-associated genes analysed in the kidneys, only 20 were significantly (P≤0.05) affected (Table 6). The genes encoding *Gstm1* and microsomal *Gst1* were significantly up-regulated by rooibos, whereas *Abcb1*, *Cyp4b1*, gamma glutamyltransferase 1 (*Ggt1*) and *Nat1* were down-regulated. *Cyclopia genistoides* up-regulated the *Cyp19a1*, *Cyp1a1* and *Hsd17β3* encoded genes, while down-regulating the genes of *Gstm3 and Gstm*5 and hexokinase 2 (*Hk2*). Similar to what was observed in the liver, *C. subternata* affected the expression of most genes. The herbal tea extract up-regulated the gene encoding of arachidonate 15-lipoxygenase (*Alox15*), aryl hydrocarbon receptor nuclear translocator (*Arnt*), *Cyp1b1*, fatty acid amide hydrolase (*Faah*), *Gstm1* and Gstm4, *Mgst2* and myeloperoxidase (*Mpo*), but up-regulated the esterase 22 (*Es22*), *Gstm5* and *Hk2* encoded genes.

Table 7 Gene expression profile of xenobiotic metabolising enzymes in kidney tissue after exposure to polyphenol-enriched herbal tea extracts for 28 days.

Gene	*A. linearis*	*C. genistoides*	*C. subternata*
Abcb1	-1.85 (P= 0.01)		
Alox15			1.71 (P= 0.05)
Arnt			1.30 (P= 0.03)
Cyp19a1		2.99 (P= 0.07)	
Cyp1a1		3.74 (P= 0.06)	
Cyp4b1	-2.19 (P= 0.05)		
Cyp1b1			5.66 (P= 0.01)
Es22			-1.23 (P= 0.04)
Faah			1.90 (P= 0.01)
Ggt1	-1.69 (P= 0.02)		
Gstm1	1.57 (P= 0.05)		1.69 (P= 0.05)
Gstm3		-1.60 (P= 0.04)	
Gstm4			1.74 (P= 0.04)
Gstm5		-1.61 (P= 0.02)	-1.69 (P= 0.01)
Hk2		-2.12 (P= 0.02)	-2.18 (P= 0.01)
Hsd17β3		4.42 (P= 0.06)	
Mgst2			2.62 (P= 0.04)
Mgst1	1.29 (P= 0.01)		
Mpo			18.60 (P= 0.05)
Nat1	-1.22 (P= 0.02)		

The values indicate fold differences.
P≤0.05 designated as significant;
- indicates down-regulation; + indicates up-regulation.
ATP binding cassette beta 1 (Abcb1), arachidonate 15-lipoxygenase (Alox15), aryl hydrocarbon receptor nuclear translocator (Arnt), cytochrome P450 19a1 (Cyp19a1), cytochrome P450 4b1 (Cyp4b1), cytochrome P450 1b1 (Cyp1b1) esterase 22 (Es22), gamma glutamyltransferase 1 (Ggt1), glutathione-S-tranferase mu (Gstm), hexokinase 2 (Hk2), 17β- hydroxysteroid dehydrogenase 3 (Hsd17β3), microsomal glutathione-S-tranferase (Mgst), myeloperoxidase (Mpo), N-acetyltransferase 1 (Nat1).

DISCUSSION

Many plant constituents are known to affect xenobiotic metabolising enzymes experimentally (Budzinski *et al*., 2000). Grapefruit juice inhibits the enzymatic activity of CYP3A4 and therefore decreases the first pass effect of the immunosuppressant, cyclosporine, leading to increased levels of the drug in the blood (Hollander *et al*., 1995). On the other hand, the herbal antidepressant, St John's wort, induces CYP450 activity and can seriously decrease cyclosporin levels (Breidenbach *et al*., 2000). In the past few decades South African herbal teas, rooibos (*A. linearis)*, and honeybush (*C. genistoides* and *C. subternata*), have become increasingly popular as beverages worldwide (Joubert *et al*., 2008). Rooibos and honeybush (*C. intermedia*) herbal teas have various effects on antioxidant capacity, immune responses, gastrointestinal actions, cancer, amongst others as reviewed by Joubert *et al*. (2008).

However, at present very little is known on the effects of these herbal teas on xenobiotic metabolism, especially when utilising polyphenol-enriched extracts. Marnewick *et al*. (2003) reported enhanced activity of glutathione-S-transferase and UDP-glucuronosyltransferase (UGT) in rat liver after intake of unfermented rooibos and *C. intermedia*. Even though no significant difference was found in total hepatic CYP content, Matsuda *et al*. (2007) showed that rooibos inhibits CYP3A-mediated MDZ-4OH activity by 80% compared to the control group in rat liver microsomes, but increased activity by 25% *in vivo* and by 50% in intestinal microsomes. These studies suggest that rooibos interferes with xenobiotic metabolism, presumably by interfering with the formation of the substrate enzyme complex. The present study represents the first

investigation of the *in vivo* effects of polyphenol-enriched extracts of rooibos and two honeybush spp, *C. genistoides* and *C. subternata* on the expression of genes encoding xenobiotic metabolising enzymes in rat liver and kidneys.

Cytochrome P450 enzymes play a pivotal role in xenobiotic as well as endobiotic metabolism in all organisms (Nebert, 1991). Approximately 75% of known drugs are metabolised by CYP450 enzymes, which are found predominantly in the endoplasmic reticulum and in greatest abundance in the liver, but are also found in extrahepatic tissues such as the kidneys, brain and intestines (Pavek and Dvorak, 2008). In the present study, the *Cyp2b6* gene was down-regulated in the liver by *C. subternata*. This enzyme is responsible for the metabolism of 4% of the top 200 drugs which includes HIV treatment drugs, efavirenz and nevirapine, and anticancer drugs, cyclophosphamide and ifosphamide (Xie *et al*., 2003; Saitoh *et al*., 2007ab). In the kidneys, the gene expressions of *Cyp1a1* and *Cyp1b1* were significantly up-regulated by *C. genistoides* and *C. subternata*, respectively, whereas *Cyp4b1* was down-regulated by rooibos. These enzymes are not only responsible for the metabolism of an abundance of drugs and other xenobiotics, but they also metabolise carcinogens such as aflatoxin B_1 and polyaromatic hydrocarbons such as benzo(α)pyrene (Gonzalez, 1990; Okey, 1990; Nebert and McKinnon, 1994; Hankinson, 1995; Beedanagari *et al*., 2010). The *Cyp1* genes are regulated by the AhR/ARNT complex (Pavek and Dvorak, 2008). Activation of the aryl hydrocarbon receptor (AhR) upon binding with its ligand causes translocation of the activated complex to the nucleus. In the nucleus, HSP90 dissociate from the activated AhR which subsequently heterodimerizes with a nuclear

transcription factor protein, the aryl hydrocarbon receptor nuclear translocator (ARNT) (Whitelaw *et al*., 1995). The AhR/ARNT complex then binds to a specific DNA recognition sequence, GCGTG, within the xenobiotic responsive element (XRE), located in the promoter region of a number of receptor regulated genes, including the CYP1A1 (Denison *et al*., 1989; Nebert *et al*., 2004). Since *C. subternata* also up-regulated the gene *Arnt* in the kidneys, it is apparent that procarcinogen metabolism may be favoured in the kidneys after exposure to the herbal tea.

The effect of these herbal teas on the expression of genes regulating steroidogenesis is of interest (Figure 2). Steroidogenic enzymes are responsible for the biosynthesis of various steroid hormones, including glucocorticoids, mineralcorticoids, progestins, and sex hormones, and consist of various specific CYPs, and several hydroxysteroid dehydrogenases and reductases (Miller, 1988). Androstenedione, a product of cholesterol catabolism, can be reversibly converted to testosterone by HSD17β3 or to estrone by Cyp19a1 (aromatase). Estrone and testosterone can be converted to estradiol by HSD17β1 and Cyp19a1, respectively (Sanderson and van den Berg, 2003) (Figure 2).

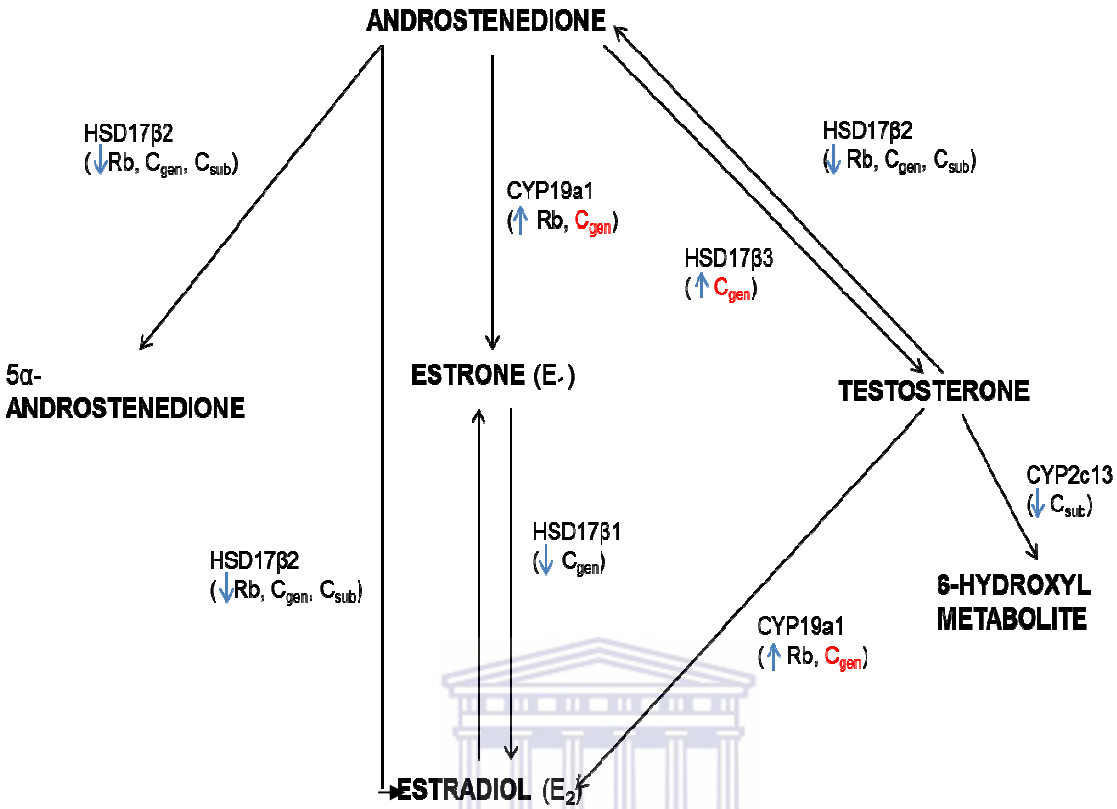


Figure 2 Schematic diagram of the enzymes involved in steroidogenesis. ↓*Indicates down-regulation,* ↑ *indicates up-regulation, Rb- rooibos,* C_{gen}*- C. genistoides,* C_{sub}*- C. subternata.* Red highlight indicates an effect in the kidneys.

The balance of steroid hormones can be affected if aromatase is targeted by xenobiotics (You, 2004). These environmental compounds can inhibit or induce aromatase gene expression or enzyme activity. A study tested aromatase activity in JAR cells (a choriocarcinoma cell line) in response to dietary polyphenols and found that green and black teas significantly decreased aromatase activity, and polyphenols inhibited estrogen production, however, no effect on aromatase expression was detected after 24 hour treatment with flavonoids (Monteiro *et al*., 2006). The flavone,

apigenin, was shown to exhibit anti-aromatase and anti-17β-hydroxysteroid dehydrogenase activity (Le Bail *et al*., 1998), however in the present study the aromatase gene expression was up-regulated by rooibos and *C. genistoides* in the liver and kidneys, respectively. Of interest is that the rooibos flavones vitexin and isovitexin, are glycosides of apigenin, however it is not known whether they will affect aromatase activity. The present findings seem to imply that the herbal tea extracts may favour the production of estradiol. This active form of estrogen is desirable in normal cells since it promotes proliferation after cell damage, however, it is not desirable in cancer cells where it would promote tumour growth (Francavilla *et al*., 1989a; Barone *et al*., 2006). Additionally, men with advanced liver diseases can develop a feminization syndrome with increased estradiol and decreased androgen levels. Similar effects are seen acutely after major liver resection (Francavilla *et al*., 1989b and 1990; Villa *et al*., 1988).

The *Hsd17β* gene expression was also altered by the herbal tea extracts; *Hsd17β1*, which catalyses the conversion of estrone to estradiol (Tremblay *et al*., 1989), was down-regulated by *C. genistoides* in the liver; *Hsd17β2*, which converts estradiol, testosterone and 5α-dihydrotestosterone to their respective less active forms, estrone, androstenedione and 5α-androstenedione (Wu *et al*., 1993), was down-regulated by rooibos, *C. subternata* and *C. genistoides* in the liver. *Hsd17β3*, which is essential for testosterone biosynthesis (Geissler *et al*., 1994), was significantly up-regulated by *C. genistoides* in the kidneys. The gene expression of *Cyp2c13*, a major cytochrome in male rat liver microsomes (Eguchi *et al*., 1991), was down-regulated by *C. subternata*. The enzyme converts testosterone to a 6β-hydroxyl metabolite (Wang *et al*., 2009).

These results indicate that polyphenol-enriched rooibos and *Cyclopia* spp. regulate the gene expression of steroidogenic enzymes by favouring estradiol anabolism in the liver and testosterone accumulation in the kidneys of rats.

Cyclopia genistoides and *C. subternata* also interfered with alcohol metabolism by favouring acetaldehyde formation in the liver, by up-regulating the genes, *Adh4* and *Cyp2e1,* respectively. However, all three herbal tea extracts significantly up-regulated aldehyde dehydrogenase (Aldh), which in the presence of NAD as a cofactor, converts the toxic acetaldehyde to less toxic acetate (Pawan, 1972; Hunt, 1996). The suggestion of an improvement to the discomfort from excessive alcohol consumption by drinking tea (*Camellia sinensis*) has been recorded by Eisai-Zenshi in the Kamakura era in Japan (Kakuda *et al*., 1996). Green tea (*C. sinensis*) extracts were shown to promote alcohol metabolism in ICR mice and to prevent the changes observed after ethanol intoxication (Skrzydlewska *et al*., 2002). This coincides with our results which indicate that rooibos and *Cyclopia* spp. may regulate ethanol toxicity at the gene expression level. However, Cyp2e1 also changes many toxicologically important substrates, including carbon tetrachloride, N-nitrosodimethylamine and acetaminophen, to more toxic products (Guengerich *et al*., 1991; Koop, 1992). The analgesic acetaminophen can be metabolically activated by Cyp2e1 to a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which depletes glutathione and covalently binds to proteins, causing a potentially fatal hepatic centrilobular necrosis (James *et al*., 2003).

Aqueous extracts of rooibos and the *Cyclopia* spp. also may have an effect on the glycolytic pathway affecting energy production. The gene expression of glucose phosphate isomerase (*Gpi*), the enzyme that catalyses the conversion of glucose-6-phosphate to fructose-6-phosphate in the second step of glycolysis, was significantly up-regulated in the liver by all three herbal teas which will entail a positive effect on glucose breakdown. On the other hand, the gene expression of hexokinase 2 (*Hk2*), an enzyme that phosphorylates glucose into glucose-6-phosphate, was down-regulated by both *C. subternata* and *C. genistoides* in the kidneys which could affect the glycolytic breakdown of glucose negatively.

The gene expressions of hepatic *Ces1* and *Ces2* were up-regulated by *C. subternata* in the liver. Carboxyl esterases are responsible for the hydrolysis of ester- and amide-bond-containing drugs (Staudinger *et al*., 2010) such as angiotensin-converting enzyme inhibitors (temocapril and cilazapril) (Takai *et al*., 1997), anti-tumor drugs (CPT-II and capecitabin) and narcotics (cocaine, heroin and meperidine) (Pindel *et al*., 1997). *Cyclopia subternata* may therefore facilitate the deactivation of these drugs. In the kidneys, the esterase gene known as egasyn (*Es22*) was down-regulated by *C. subternata*. The enzyme specifically hydrolyses retinyl esters to form retinol (Mentlein and Heymann, 1987), which is the most active form of animal derived vitamin A. Vitamin A is essential for vision, bone growth and immune responses amongst others (D'Ambrosio *et al*., 2011). Therefore an inhibition of Es22 attenuates an accumulation of retinol esters (van Berkel, 2009), thus providing a precursor for vitamin A synthesis.

Cyclopia subternata up-regulated the arachidonate 15-lipoxygenase (*Alox15*) gene in the kidneys. *Alox15* is responsible for the synthesis of 15S-hydroperoxyeicosatetraenoic (15-HETE) acid by region-selectively adding oxygen to arachidonic acid. 15-HETE decreases renal blood flow and glomerular filtration (Imig, 2000) and it has anti-proliferative and pro-apoptotic functions (Kumar *et al*., 2009). *Cyclopia subternata* may therefore impede on the elimination of drugs, but also protect against cancer development.

Fatty acid amide hydrolase (*Faah)* gene was up-regulated by *C. subternata* in the kidneys. This gene encodes a protein that is responsible for the hydrolysis of a number of primary and secondary fatty acid amides, including neuromodulatory compounds, anandamide (Devane *et al*., 1992) and oleamide (Cravatt *et al*., 1995), thereby serving to eliminate the signaling functions of the molecules. Inactivation of Faah has been linked to sleep enhancement (Huitron-Resendiz *et al*., 2004) due to the lack of anandamide degradation (Murillo-Rodriguez *et al*., 2007), anti-depressant (Cravatt *et al*., 2004) and anti-inflammatory (Lichtman *et al*., 2004; Cravatt *et al*., 2004; Holt *et al*., 2005) effects due to elevated endogenous levels of fatty acid amides. *Cyclopia subternata* may therefore restrain sleep, and induce depression and pain caused by inflammation.

The significant up-regulation of the myeloperoxidase *Mpo* gene in the kidneys by *C. subternata* is of interest. Mpo, a member of the haem peroxidase-cyclooxygenase family, is abundantly expressed in neutrophils and to lesser extent in monocytes and

certain types of macrophages. During inflammation Mpo is released from cytoplasmic granules of neutrophils by a degranulation process, where it reacts with hydrogen peroxide (H_2O_2) to form a complex that can oxidise a large variety of substances (Klebanoff, 2005). Mpo-H_2O_2 can react with chloride leading to the formation of reactive oxidants (hypochlorous acid) which is important in the innate immune defenses. However, if released outside the cell, it may induce damage to adjacent tissues (Malle *et al*., 2007). It has been suggested that renal glomerular damage may be caused by the Mpo system. Mpo has also been associated with pyelonephritis, membrane proliferative glomerular nephritis and membranous glomerular nephritis (Odobasic *et al*., 2007). It has been shown that Mpo metabolises the anticonvulsant carbamazepine to reactive intermediates that could be responsible for the adverse reactions (such as agranulocytosis and lupus) associated with the drug (Furst and Uetrecht, 1993).

The gene expression of phase II enzymes, specifically the glutathione tranferases and N-acetyltransferase, were altered in the kidneys after exposure to all the herbal teas. Marnewick *et al.* (2003) found that unfermented rooibos and *C. intermedia* significantly enhanced the activity of cytosolic glutathione S-transferase alpha in the rat liver, however, in the present study no effect on the gene expression of the enzyme in the liver was noticed. In addition, the gene encoding gamma glutamyltransferase (*Ggt1*), an enzyme involved in the degradation of glutathione, was down-regulated by rooibos in the kidneys. Glutathione degradation occurs in the proximal tubule at both the luminal (Hahn *et al*., 1978) and basolateral membrane (Abbot *et al*., 1984) a process that is modulated by rooibos presumably due to changes in GSH metabolism in the body.

Finally, of the phase III drug transporters, the ATP-binding cassette (ABC) transporters constitute a ubiquitous superfamily of integral membrane proteins that are responsible for the translocation of many substances across membranes. Rooibos down-regulated the *Abcb1* gene in the kidneys, which could be advantageous in the treatment of cancer during chemotherapy, however the protection of normal cells against toxic compounds may be impaired. This also indicates that rooibos polyphenols may prevent their own transport out of the kidney cells.

Differences in the polyphenol intake between the herbal teas may explain the differential expression of the genes in the liver and kidneys. At present very little information is available on the effect of polyphenols on the expression of xenobiotics metabolising enzymes in the liver and kidney. The highest polyphenol intake was obtained in the rats receiving the rooibos extract with the dihydrochalcone aspalathin as the major polyphenol. Of the honeybush herbal teas, rats receiving the *C. subternata* extract had the highest polyphenol intake despite the fact that the mangiferin content was up to two fold lower than *C. genistoides*. However, the exposure to the flavanones, hesperidin, eriocitrin eriodictyol glycoside and the flavones, specifically scolymoside was significantly higher when compared to the rats receiving the *C. genistoides* extract. In the liver only three genes were affected by all three herbal teas with the flavone, luteolin the only common flavonoid present. It is not known at present whether luteolin will modulate the expression of Ald1a1, Gpi and *Hsd17β2*. In the kidney, however the expression of very few genes is simultaneously altered by the herbal teas which could be related to the presence of different metabolic and/or conjugated forms of the

polyphenols. Of interest is the *Cyclopia subternata* affected more of the genes compared to *C. genistoides* and rooibos in both the liver and kidneys while it also significantly enhanced the total bilirubin serum levels. This coincided with a higher total polyphenol intake when compared to *C. genistoides* although it was significantly lower than the rooibos polyphenol intake. It would appear that specific or different combinations of polyphenols and/or non-polyphenolic constituents and their respective metabolites could be involved in the differential expression of the xenobiotic metabolising enzymes in the body.

The current investigation indicated herbal teas differentially altered the gene expression of xenobiotic metabolising enzymes in the liver and kidneys of male Fischer rats, suggesting that rooibos and honeybush herbal teas can restrain and/or enhance the metabolism of both foreign and endogenous compounds. Investigation of the effect of rooibos and *Cyclopia* spp. polyphenols on the protein expression levels of the altered genes and enzyme interactions may provide insight into the downstream effects.

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CHAPTER 4

Validation of the rat primary hepatocyte model to predict *in vivo* effects of polyphenol-enriched herbal tea extracts and the major polyphenols, aspalathin and mangiferin, on the gene expression of selected xenobiotic metabolising enzymes

ABSTRACT

In vivo studies are often expensive and labour intensive, therefore scientists are exploring other methods that can mimic the effects observed *in vivo*. The current study investigated whether or not rat primary hepatocytes may serve as a tool to predict the effects of polyphenol-enriched extracts on the gene expression of xenobiotic metabolising enzymes in the liver. Isolated rat primary hepatocytes were exposed to non-toxic concentrations of extracts of aspalathin-enriched rooibos and mangiferin-enriched *C. subternata* and *C. genistoides*, and their major polyphenolic constituents, aspalathin and mangiferin, respectively for 24 hours. The gene expression of selected xenobiotic metabolising enzymes were analysed by quantitative real-time PCR.

*C*yclopia *subternata* significantly (P≤0.05) down-regulated the *Comt* gene with mangiferin down-regulating both *Comt* and the *Hsd17β2* genes. Aspalathin up-regulated the *Comt* and marginally (P<0.1) the *Cyp2e1* genes, and down-regulated the *Hsd17β2* gene. *Cyclopia genistoides* markedly decreased the expression of all three genes while rooibos slightly reduced the expression of *Comt*. However, the changes were not statistically significant. The results differ from the *in vivo* study in that the *Comt* gene was affected by *C. subternata* while the other herbal teas markedly reduced the gene expression of *Cyp2e1* and *Hsd17β2*. Primary hepatocytes seems to be an appropriate model to mimic changes in the expression of *Hsd17β2* gene by the herbal teas with aspalathin and mangiferin to be the likely candidates to play a role. However, differences exist depending on the specific gene under study when considering the effect of a pure polyphenol and the complex enriched polyphenol extracts. Additional studies, investigating the effect of different doses of the herbal teas, combination of

different polyphenolic constituents and a longer exposure period is recommended to further validate the *in vitro* rat primary hepatocytes as a model to predict changes observed *in vivo*.

Keywords: Aspalathin, mangiferin, rat primary hepatocytes, xenobiotic metabolising enzymes.

INTRODUCTION

Living organisms, with their extremely complex functional systems and cell interactions, are difficult to study *in vivo*. Thousands of genes, protein molecules, nucleic acids and other components are spatially organised by membranes or organ systems, which creates a barrier for the identification of individual components and the exploration of their basic biological functions. *In vitro* studies allow scientists to overcome these barriers by isolating components from their biological context to permit a more detailed and convenient applicable analyses that could otherwise not have been conducted utilising the whole organisms (Chenery *et al*., 1987; Le Bigot *et al*., 1987; O'Brien *et al*., 2004).

Seventy to 80% of the liver's mass consist of hepatocytes that are involved in various biological processes such as protein synthesis and storage, transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, but of more importance is its role in the detoxification, modification and excretion of exogenous (xenobiotic) substances (Orzechowski *et al*., 1995; Bort *et al*., 1999). Intestinal venous blood and xenobiotics drained into the liver are detoxified by enzymes to maintain homeostasis and to protect the organism against the adverse effects of ingested toxins. The xenobiotic metabolising enzymes modify the xenobiotics in order to facilitate their excretion by either adding or unveiling a polar functional group. Primary rat hepatocytes retain the activity of the majority of these xenobiotic metabolising enzymes and therefore provide an excellent model to investigate xenobiotic metabolism *in vitro* (Paillard *et al*., 1999).

In the previous chapter, an *in vivo* study indicated that polyphenol-enriched extracts of rooibos and two honeybush species, *C. subternata* and *C. genistoides*, altered the gene expression of several xenobiotic metabolising enzymes in the liver and kidneys of male Fischer 344 rats after exposure for 28 days. The current study investigated the effect of the same polyphenol-enriched extracts, as well as the major polyphenol constituents of rooibos and honeybush, aspalathin and mangiferin, respectively on the gene expression of selected genes coding for xenobiotic metabolising enzymes in rat primary hepatocytes. The genes selected were based on the findings that the three herbal tea extracts significantly down-regulated the hydroxysteroid dehydrogenase (*Hsd17β2*) gene, but did not significantly alter the cathechol-O-methyltransferase (*Comt*) gene while the cytochrome P450 (*Cyp2e1*) gene was only slightly but significantly up-regulated by *C. subternata*. A comparison of the results to that obtained *in vivo* will be conducted to validate whether the *in vitro* findings, utilising the rat primary hepatocyte model, can be extrapolated to the effects observed *in vivo.*

MATERIALS AND METHODS

Chemicals

Aspalathin-enriched green rooibos (18.7% aspalathin), mangiferin-enriched green *C. subternata* (3.8% mangiferin) and *C. genistoides* (9.3% mangiferin) extracts were supplied by the RAPS Foundation (Germany). Aspalathin was purified from unfermented rooibos at the PROMEC Unit of the Medical Research Council, Tygerberg, South Africa to a purity of >95% and mangiferin was obtained from Sigma-Aldrich (Cape Town, South Africa).

The Williams' E medium (WE), Hanks buffer salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), rat tail collagen type 1, collagenase type 4, insulin, sodium-pyruvic acid, L-glutamine, L-proline, ethylene glycol tetraacetic acid (EGTA), dimethyl-sulfoxide (DMSO) and Eagle's minimal essential medium (EMEM) were purchased from Sigma-Aldrich (Cape Town, South Africa) as was the molecular biology grade β-mercaptoethanol (β-ME). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA). Penicillin, streptomycin, fungizone (Amphotericin B), trypan blue and MEM non-essential amino acid solution were obtained from BioWhittaker/Lonza (Switzerland). Analytical grade methanol, ethanol and hydrochloric acid, were purchased from Merck Chemicals (Johannesburg, South Africa).

Chemical analysis of herbal tea samples

The determination of the total and individual polyphenol content of the enriched herbal tea extracts, by HPLC is described in Chapter 3.

Animals

The study was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council (MRC). Male Fischer 344 rats, 7-8 weeks old and weighing between 150-200g, were obtained from the Primate Unit of the Medical Research Council of South Africa. The animals were housed in stainless steel wire-bottomed cages in a room illumination of 12 hr photoperiods at 50% humidity and a constant room temperature of 24-25°C. Rats had free access to food (Epol Ltd, South Africa) and water. Prior to termination, rats were anaesthetised by injecting 0.22 mg per 100g body weight sodium pentobarbital intraperitoneally.

Preparation of rat primary hepatocyte cultures

Primary hepatocytes were isolated from male Fischer 344 rats according to the collagenase perfusion technique as described by Hayes *et al*. (1984) (See addendum III for detailed protocol). Briefly, in a sterile environment, the abdomen of the rat was opened through a midline incision and the liver was exposed after dissecting the surrounding structures. A cannula was then placed in the portal vein and the liver was perfused with 10% HBSS (containing 1 % EGTA) at 37°C, at a flow rate of 15 ml per minute for 10 min. Subsequently, a collagenase solution (37°C) was circulated through the liver at a flow rate of 4-5 ml/min for approximately 13 min. The liver was removed and placed into a petri dish filled with collagenase solution. Modified WE medium, containing 10% FBS was immediately added to inactivate the enzyme. The liver capsule was removed and the cells were harvested in the medium. The resulting cell suspension was filtered through a sterile single layer cheesecloth and washed with WE containing 10% FBS at 450 rpm for 10 minutes. After removing the supernatant, WE containing

10% FBS was added to the pellet and the cell suspension filtered through double layered cheesecloth. Prior to plating, the percentage cell viability was determined using trypan blue stain and a haemocytometer. Only isolates yielding a viability of 80% and greater were used for future experiments. Cells were plated at a density of 3×10^6 viable cells in collagen-coated culture dishes (60 mm) for RNA extractions or 2×10^4 viable cells in collagen-coated 96-well opaque-walled plates for cell viability assays and incubated in WE medium containing 10% FBS, insulin (20 U/L), L- glutamine (2 mM), HEPES (10 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) for 3 hrs at 37℃ to allow cell attachment. The cells were then washed twice with HBSS and supplemented with modified WE medium (200 µl in 96-well plates or 2 ml in 60 mm plates) containing 0.5% FBS, L- proline (2 mM) and sodium pyruvate (10 mM), in addition to the other components described above, and incubated for 24 hrs at 37℃ in air/carbon dioxide (95:5).

Cell viability assay to determine IC_{50}'s

DMSO solubility of herbal tea extracts, aspalathin and mangiferin

The herbal tea extracts and polyphenols were first dissolved in 10% and 100% DMSO, respectively (Table 1), to yield a stock solution, and then diluted in culture medium (modified WE) to create a 1% DMSO concentration in each dilution to be used in the cell culture studies.

Table 1 Solubility of herbal tea extracts, aspalathin and mangiferin in various concentrations of DMSO.

Extract/ compound	DMSO percentiles				
	Pure/ 100%	**10%**	**5%**	**1%**	**0.5%**
Rooibos	N.A	Soluble	Soluble	Insoluble	Insoluble
C. genistoides	N.A	Soluble	Soluble	Insoluble	Insoluble
C. subternata	N.A	Soluble	Soluble	Insoluble	Insoluble
Aspalathin (dihydrochalcone)	Soluble	Insoluble	Insoluble	Insoluble	Insoluble
Mangiferin (xanthone)	Soluble	Insoluble	Insoluble	Insoluble	Insoluble

N.A (not applicable) indicates that it has not been analysed.

Treatment with extracts, aspalathin and mangiferin

Following incubation of the hepatocytes, the medium was removed and the cells washed with HBSS. Different concentrations of the polyphenol-enriched extracts or polyphenol (100 µl) was added to the respective wells and the 96-well opaque-walled microtitre plate incubated for 24 hrs at 37℃ in air/carbon dioxide (95:5). The dilutions used for the rooibos extracts were 0.2, 0.1, 0.05, 0.025 mg/ml and 2.0, 1.5, 1.0, 0.5 mg/ml for *C. subternata* and *C. genistoides*. Different dilutions for aspalathin (2.0, 1.25, 0.63, 0.31, 0.16 µM) and mangiferin (1.25, 1.00, 0.63, 0.31 µM) were prepared in DMSO (1%). A 1% DMSO solution in culture medium was used as a control sample.

Cell viability assay

The number of viable cells was determined based on the assessment of ATP production as a measure of metabolically active cells, using the CellTitre-Glo® assay (Promega,

Wisconsin, USA). The plates and the assay kit components were kept at room temperature prior to performing the assay. The assay was conducted at room temperature according to the manufacturer's protocol. Briefly, the CellTitre-Glo® reagent (100 µl) was added to each well containing the test compounds and controls and the contents mixed on a shaker for 2 min and incubated for 10 min. The luminescence was recorded using a Veritas™ Microplate Luminometer (Promega, Wisconsin, USA). The percentage ATP inhibition was determined and the respective IC_{50} values (concentration of test compound required to inhibit 50% ATP production in the cell) were calculated using the PRISM® 5 programme. The assay was repeated five times to obtain an average IC_{50} value for each extract and/or pure compound.

Gene expression experiments

Incubation conditions

Cells in the 60 mm culture dishes were exposed to IC_{50} levels (Table 3) and at non-toxic levels (≥80% viability) of rooibos (0.025 mg/ml), *C. genistoides* (0.5 mg/ml), *C. subternata* (0.5 mg/ml), aspalathin (0.31 µM) and mangiferin (1.25 µM) for 24 hrs at 37℃ in air/carbon dioxide (95:5). Each treatment and control were repeated four times in a total volume of 2 ml per tissue culture dish

Cell disruption

The hepatocyte cultures were washed with ice cold phosphate buffered saline (pH=7.4), scraped in RLT buffer* (350 µl/dish), containing guanidine isothiocyanate and β-

mercaptoethanol, pooled (total of 4 dishes) into clean nuclease-free tubes and then disrupted in liquid nitrogen.

**The description, concentration and pH of the buffer are not defined in the RNA extraction kit user manual.*

Total RNA isolation, quality assessment and reverse transcription

Total RNA was isolated from the hepatocyte cultures, the quality was assessed and the RNA was reversed transcribed into cDNA, as previously described in Chapter 3.

Primer design and optimisation

The gene expression of three xenobiotic metabolising genes were analysed by qRT-PCR. The hypoxanthine guanine phosphoribosyltrasferase (*Hprt1*) gene was used as an endogenous control. Gene- specific qPCR primers (Table 2) were designed using the Primer Express® software v2.5 (Applied biosystems, South Africa) and synthesized by Integrated DNA technologies (IDT, Whitehead scientific, South Africa).

Table 2 Primer sequences of selected genes for qPCR analyses.

Gene	Genebank	Sense	Antisense
		5'→3'	**5'→3'**
Hsd17β2	NM_024391	CGCAGAGAAGATACAAGACAAAGG	AAAGTGCAAGACCCCAGCAT
Comt	NM_012531	CCATGGAGATGAACCCTGACTAC	TAGGCCTGCAAAGTTCAGCATT
Cyp2e1	NM_031543	TTGGCCGACCTGTTCTTTG	TTTCTGGGTATTTCATGAGGATCA
Hprt1	NM_012583	TCAAGCAGTACAGCCCCAAAAT	CAACACTTCGAGAGGTCCTTTTC

Hsd17β2- 17β-Hydroxysteroid dehydrogenase 2, Comt- Catechol-O-methyltransferase, Cyp2e1- Cytochrome P450 2e1, Hprt- Hypoxanthine guanine phosphoribosyl transferase.

Quantitative real-time PCR (qPCR)

The qPCR and statistical analyses are described in Chapter 3.

RESULTS

Dose response effects

Herbal tea extracts

Typical dose response effects were observed on cell viability for each of the herbal tea extracts (Figure 1). Compared to the rooibos extract, up to ten times the concentration of the honeybush extracts was needed for obtaining dose response effects.

Comparative effects of Cyclopia extracts

A comparison of the dose response effects of the *Cyclopia* extracts revealed that *C. subternata* inhibited ATP production at a significantly lower concentration than *C. genistoides* (Figure 2).

Individual polyphenols

Typical dose response effects were observed for aspalathin, whereas the dose response for mangiferin could not be obtained at the concentrations used. This was due to insolubility of mangiferin in the cell culture medium at higher concentrations (Figure 3).

IC_{50} value of extracts, aspalathin and mangiferin

The IC_{50} for cytotoxicity for rooibos was significantly ($P<0.05$) lower when compared to that of the honeybush herbal teas (Table 3). Of the honeybush herbal teas *C. subternata* exhibited a higher cytotoxic effect despite the fact that the TP content was lower. Aspalathin exhbited an IC_{50} of 1.91 µM while no value could be obtained for mangiferin due to its insolubility at high concentrations.

Table 3 Concentration of rooibos, *C. genistoides*, *C. subternata*, aspalathin and mangiferin required to inhibit 50% ATP production in rat primary hepatocytes.

Extract	***IC_{50}***	***TP equivalents***
A. linearis	0.12 ± 0.01mg/ml[a]	47.1
C. genistoides	1.52± 0.17mg/ml[b]	332.6
C. subternata	1.12 ± 0.09mg/ml[c]	278.1
Pure polyphenols		
Aspalathin	1.91 ± 0.05µM	
Mangiferin	>1.25 µM*	

*Means ± std of two experiments. Means followed by the same letter do not differ significantly, if letter differs then P<0.05). *Insoluble at higher concentrations.TP- total polyphenols. IC_{50} - inhibition of 50% of ATP production.*

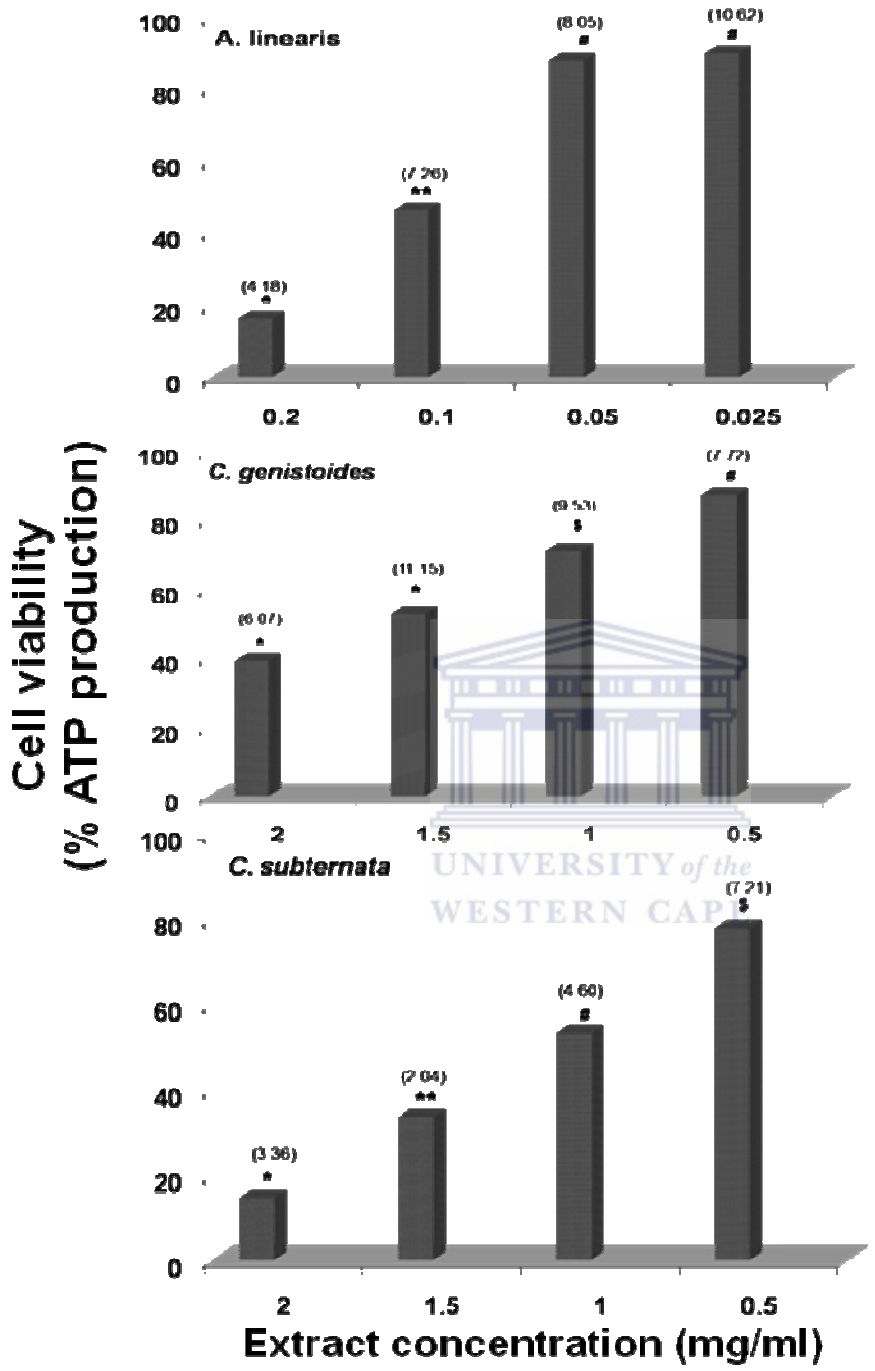

Figure 1 Dose response effects of *A. linearis*, *C. genistoides* and *C. subternata* on ATP production in rat primary hepatocytes. Values are means ± Std (values in parenthesis) of at least two experiments. Different symbols on the bars indicate significant differences (P≤0.05).

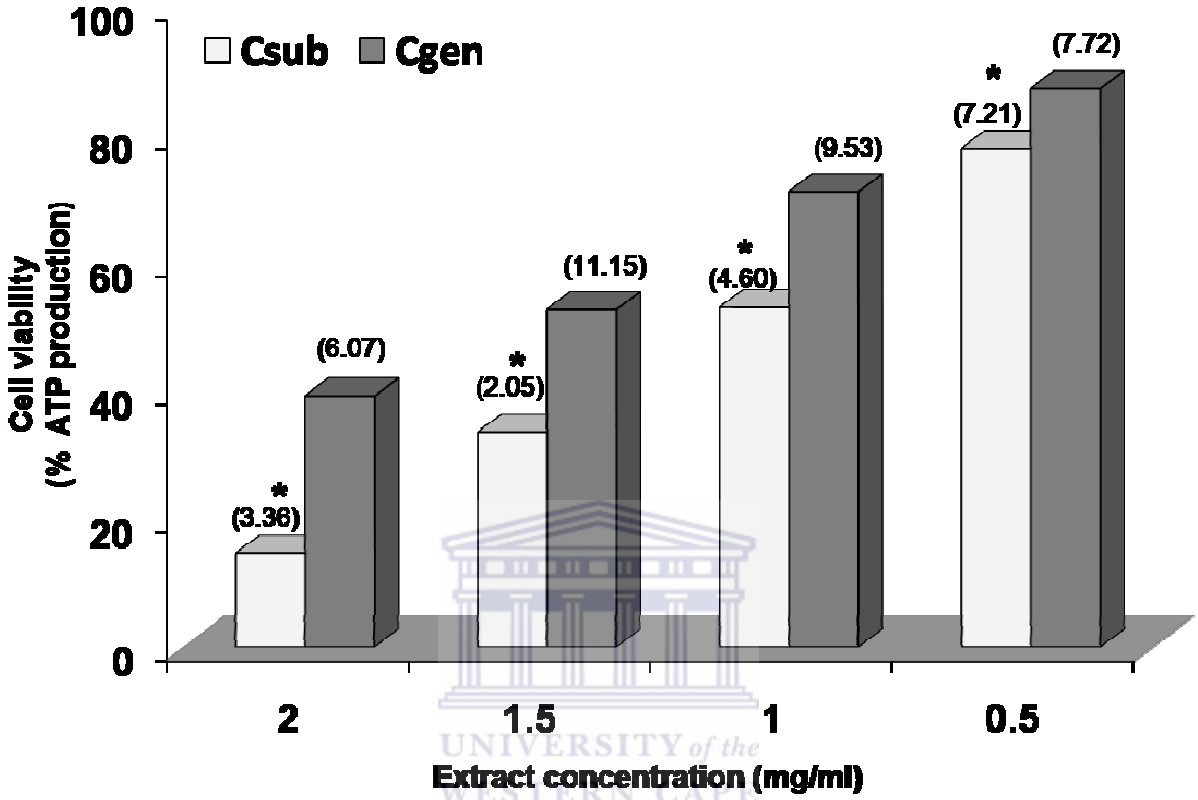

Figure 2 Comparison of the dose response effects of *Cyclopia spp.* on ATP production in rat primary hepatocytes. Values are means ± Std (values in parenthesis) of at least two experiments. *indicate significant difference (P≤0.05).

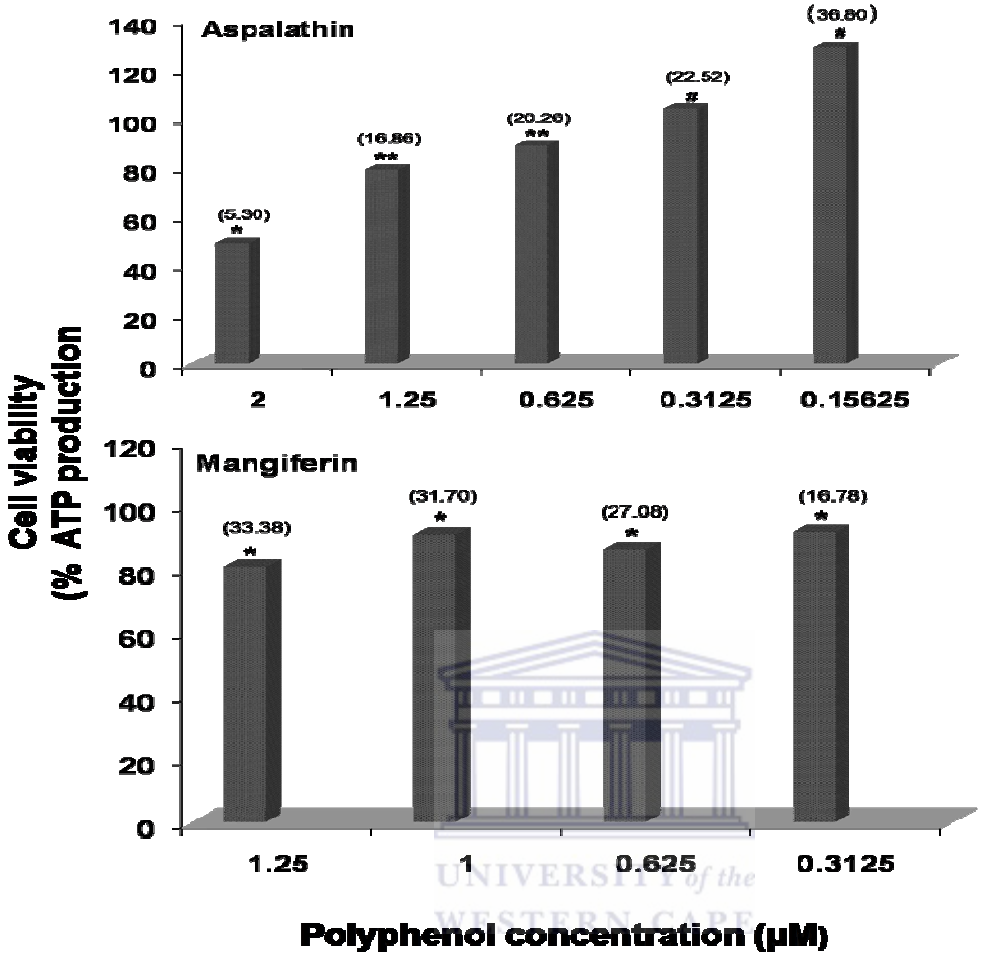

Figure 3 Dose response effects of aspalathin and mangiferin on ATP production in rat primary hepatocytes. Values are means ± Std (values in parenthesis) of at least two experiments. Different symbols on the bars indicate significant differences (P≤0.05).

Concentration of TPP and monomeric flavonoids utilised for gene expression analyses

Rooibos herbal tea (A. linearis)

The dihydrochalcone aspalathin was the major flavonoid in the rooibos extract that was added to the hepatocytes followed by rutin+Isoquercitrin+Quercetin-3-robinobioside, nothofagin, isoorientin and orientin (Table 4). Only small amounts of vitexin, isovitexin,

hyperoside, luteolin-7-glucoside, luteolin, chrysoeriol were recorded as well as traces of quercetin. The TPP level of rooibos used was similar to that of *C. genistoides*.

Honeybush herbal teas (*Cyclopia spp.*)

Of the honeybush polyphenolic constituents added to the hepatocytes, the xanthones mangiferin and isomangiferin were found to be the most abundant (Table 4). *Cyclopia genistoides* had a higher xanthone content, whereas *C. subternata* contained a higher flavanone content due to the presence of eriocitrin. The level of hesperidin, naringenin and eriodictyol and the flavone, luteolin were similar while scolymoside was higher in *C. subternata* (Table 4). When considering the TP content, the hepatocytes were exposed to a higher level when using the *C. subternata* extract.

Gene expression analysis by qRT-PCR

Athough test samples at IC_{50} level were initially included in the experiment, it generated too little RNA due to the low number of the cells resulted from cytotoxic effects, and therefore only the results of the non-toxic levels are reported.

Table 4 Monomeric rooibos and honeybush polyphenolic constituents of the enriched extracts exposed to the cells in the culture dishes.

Aspalathus linearis (Rooibos)

Polyphenol content	***Compound***	***µg/0.025 mg extract****
TP	-	98.1
Dihydrochalcone	Aspalathin	4.70
	Nothofagin	0.36
Flavone *C*-glycoside	Isoorientin	0.36
	Orientin	0.20
	Vitexin	0.075
	Isovitexin	0.098
Flavonol	Rutin+Isoquercitrin+Quercetin-3-robinobioside	0.53
	Quercetin	Traces
	Hyperoside	0.073
Flavone	Luteolin	0.02
	Chrysoeriol	0.005
	Luteolin-7-glucoside	0.01

Cyclopia spp. (Honeybush)

Polyphenol content	***Compound***	***C. genistoides µg/0.5 mg extract****	***C. subternata µg/0.5 mg extract****
TP	-	109.4	124.2
Xanthones	Mangiferin	46.5	19.0
	Isomangiferin	16.5	6.5
Flavanones	Hesperidin	4.15	4.95
	Eriocitrin	nd	5.50
	Naringenin	0.05	0.05
	Eriodictyol-glucoside	0.15	1.15
Flavone	Luteolin	0.45	0.3
	Scolymoside	nd	6.45

*The data represents the amount of monomeric polyphenols exposed to cells in the culture dish and is based on the HPLC data summarised in Chapter 3, Table 2. nd- not detected. *concentration of extract associated with ≥80% cell viability after 24 hrs. TP – total polyphenols.*

Rooibos and *C. genistoides* did not significantly alter the gene expression of the analysed genes, while *C. subternata* significantly down-regulated the *Comt* gene, (Table 5). However, rooibos markedly reduced the expression of COMT, while *C. genistoides* reduced the expression of *Hsd17β2 and Cyp2e1*, although changes were not significant. Aspalathin significantly (P≤0.05) increased the expression of *Comt* and down-regulated the *HSD17β2* gene while mangiferin down-regulated both the *Comt* and *Hsd17β2* genes by 50%. Aspalathin also marginally increased the expression of *Cyp2e1*.

Table 5 Gene expression induction/inhibition of xenobiotic metabolising enzymes after exposure to various test samples at non-toxic levels.

Treatment	*Comt*		*Hsd17β2*		*Cyp2e1*	
	Fold	**P value**	**Fold**	**P value**	**Fold**	**P value**
A. linearis	0.83	0.15	1.18	0.62	1.10	0.21
C. genistoides	0.67	0.36	0.64	0.64	0.77	0.52
C. subternata	0.76	0.05	0.77	0.46	0.83	0.39
Aspalathin	2.05	0.04	0.68	0.05	1.65	0.07
Mangiferin	0.55	0.002	0.54	0.05	0.98	0.15

Data normalised against the Hprt1 reference gene. Control (untreated) group set as fold=1. Comt= catechol-o-methyltransferase, Cyp2e1= cytochrome P450 2e1, Hsd17β2= 17-beta hydroxysteroid dehydrogenase type 2. Shaded areas – significant changes (P≤0.05); Lightly shaded areas – marginal (P<0.1)/marked reduction. Concentration of herbal tea extract per culture dish: A linearis (0.25 mg/ml) Cyclopia spp (0.5 mg/ml); Aspalathin (0.31 µM) and mangiferin (125 µM).

DISCUSSION

The liver plays an essential role in the biotransformation of endogenous molecules as well as xenobiotics, including pharmaceutical drugs, plant polyphenols and procarcinogens (Pineiro-Carrero and Pineiro, 2004). Since metabolic enzymes occur in abundance in the liver, it is the primary organ used in pharmacological and toxicological studies (Wilkening *et al*., 2003). Although *in vivo* models provide a fairly accurate indication of the effects of chemical compounds on a whole animal, experiments are usually time- consuming, labour intensive and expensive. *In vitro* cell models are therefore employed not only to minimise cost and time, but also to elucidate specific mechanisms of action. Because of the predominance of hepatocytes, in terms of abundance and functional contribution to the liver, primary hepatocyte cultures, have been utilised as a prominent tool for *in vitro* studies (Brandon *et al*., 2003). It is a well-known model for investigating drug metabolism, induction of drug metabolising enzymes and cytotoxicity studies of chemicals (Wang *et al*., 2002). Both cultured hepatocytes and suspensions of primary hepatocytes have been repeatedly proven to be powerful tools to analyse the specific metabolic profile of a variety of drugs with good *in vitro-in vivo* correlations (Le Bigot *et al*., 1987; Chenery *et al*., 1987; Berry *et al*., 1992; Cross *et al*., 1995; Bayliss *et al*., 1999; O'Brien *et al*., 2004). The disadvantages of using primary hepatocytes are, however, that proliferation does not occur, cells only remain viable for up to 4 weeks in culture (Brandon *et al*., 2003; Wilkening *et al*., 2003) and they are subjected to a gradual loss of liver-specific functions, with special reference to a decreased cytochrome P450 expression (George *et al*., 1997). It also lacks non-

hepatocyte cells such as Kupffer cells that may be necessary for cofactor supply (Brandon *et al*., 2003).

The current study used rat primary hepatocytes as an *in vitro* cell model to investigate differences in the gene expression of three selected xenobiotic metabolising enzymes in rat liver by exposing the cells to polyphenol-enriched extracts rooibos and honeybush and their major polyphenols, aspalathin and mangiferin, respectively. Although the *Comt* gene, whose enzyme is known to metabolise tea polyphenols having a catechol moiety, was not significantly affected by the herbal tea extracts in the *in vivo* study, it was significantly down-regulated by *C. subternata* and mangiferin, and up-regulated by aspalathin in the primary hepatocytes. Studies reported that catechol-containing tea polyphenols are rapidly *O*-methylated by the COMT enzyme (Morand *et al*., 1998; Zhu *et al*., 1994) and other studies suggest that the biological action of tea polyphenols may be attributed to its methylated products (Zhu *et al*., 2000; Huffman *et al*., 2003). Polyphenols also inhibits COMT-mediated *O*-methylation of catechol estrogens (Zhu *et al*., 2009), due to its higher affinity for the enzyme (Manach *et al*., 2004). Polyphenols are known to mimic the effects of catechol estrogens by competing for the estrogen receptor β binding sites (Mfenyana *et al*., 2008; Martin *et al*., 1996; Loukovaara *et al*., 1995; Makela *et al*., 1995; Keung, 1995; Hu *et al*., 2010).

Hsd17β2, the gene that codes the enzyme responsible for the catabolism of testosterone and the anabolism of estradiol (E_2), was significantly down-regulated by all three herbal tea extracts in the *in vivo* study. In primary hepatocytes aspalathin and mangiferin significantly down-regulated the gene, while both the honeybush herbal teas

markedly reduced the level of expression. The *Cyp2e1* gene was not significantly altered by the herbal tea extracts although the expression was markedly reduced by both *Cyclopia* spp. This also contradicts the *in vivo* findings where *C. subternata* significantly up-regulated the gene by 1.23 fold. In the current study aspalathin significant up-regulated the expression of *Cyp2e1* while no effect was noticed with the rooibos extract.

This study showed a good *in vitro* and *in vivo* comparison with respect to the *Hsd17β2* gene, however the *Comt* and *Cyp2e1* genes did not correspond with the *in vivo* findings. Therefore, caution should be observed when using rat primary hepatocytes to validate genes *in vivo*. When conducting *in vitro* studies for toxicity or metabolism evaluation, especially when using primary hepatocytes, it should be noted that the concentration of the extracts/pure compound used are relatively low and exposure occur over a far shorter period of time than what would normally occur in *in vivo* studies. An example of this is that, in the previous *in vivo* study (Chapter 3), animals were exposed to a rooibos extract containing 36 mg aspalathin per 100 g rat bodyweight per day for 28 days, while in the present *in vitro* study, cells (3×10^6) were exposed to the rooibos extract (0.025 mg/ml), containing 4.7 µg aspalathin, for 24 hours. The *in vivo* exposure to rooibos TP was significantly higher when compared to the honeybush herbal teas. In the primary hepatocyte model, however, the TP exposure to the cells using the rooibos and honeybush extracts were similar, except for *C. subternata* which was slightly higher. Therefore, cognisance of the different exposure regimens has to be taken when comparing the effect on the gene expression between the two models. This could

explain why differences were observed in the expression of the xenobiotics metabolising genes between the *in vivo* and *in vitro* models.

Additionally, *in vivo* effects could be due to metabolic conjugates, formed during metabolism in other organ systems such as the intestines, rather than the parent polyphenol (Kroon *et al*., 2004). It is imperative to note that polyphenols are substantially modified during first-pass metabolism and are therefore molecularly different from those present in foods, when reaching the target tissues (Day *et al*., 2001; Day and Williamson, 2001; Graefe *et al*., 2001; Natsume *et al*., 2003; Setchell *et al*., 2003; Zhang *et al*., 2003). The resulting metabolites formed in the intestines are typically glucuronate and sulfate conjugates, with or without methylation across the catechol functional group (Kroon *et al*., 2004). The hepatocytes are exposed to the conjugates via the blood in the hepatic portal vein and may further metabolise them via phase II metabolism. The conjugates are chemically distinct from their parent compounds, differing in size, polarity, and ionic form. Consequently, their physiological behaviour is likely to be different. In contrast, when conducting *in vitro* studies, the cultured hepatocytes are exposed to polyphenols that have not been subjected to metabolism. *In vivo* effects of polyphenols could therefore be masked when conducting *in vitro* studies to predict *in vivo* scenarios. It is thus necessary to identify their metabolites and test their own biological activity (Scalbert *et al*., 2002).

Another explanation for the variation in *in vivo* and *in vitro* effects is that differences exist when cells are exposed to a single substance and/or polyphenol or towards a complex mixture containing polyphenolic and non-polyphenolic constituents as was

conducted in the current experiment. When considering aspalathin, the hepatocyte cultures were exposed to 0.28 µg (0.31 µM) of the pure dihydrochalcone while a 16 fold higher level was achieved when using the polyphenol-enriched rooibos extract. A similar effect was noticed with mangiferin (1.06 µg) where a 20 to 40 fold higher concentration was achieved when using the enriched extracts of *C. subternata* (19.0 µg) and *C. genistoides* (46.5 µg), respectively. It would appear that the effects in primary hepatocytes of the pure polyphenols were masked when utilising the polyphenol-enriched extracts. In this regard it was shown that when utilising a rat uterotrophic assay individual estrogens gave an opposite effect as compared to when tested as a mixture (Tinwell and Ashby, 2004). However, additive effects of single and combinations of compounds could also occur depending on the specific model used. In caco-2 colon cell monolayers, individual polyphenols increased the apical transport of a pro carcinogen while a complex mixture of different polyphenols increased the absorption (Schutte *et al*., 2008). In the current study both aspalathin and mangiferin significantly down-regulated the expression of *Hsd17β2* in primary hepatocytes while only marked effects were noticed with the polyphenol-enriched extracts. It could be argued that different polyphenols may have opposite effects on the expression of the gene *in vitro* while other parameters, such as bioavailability and metabolism could modulate the effect of the herbal teas on the expression of the gene differently. It would therefore be of interest to monitor the effect of aspalathin on the expression of *Hsd17β2 in vivo*. However, the current study implies that both aspalathin and mangiferin may play an important role in the down-regulation of steroid catabolism *in vivo* which should be further investigated.

CONCLUSIONS

Differences as well as similarities were observed between the *in vivo* and *in vitro* studies. This could be due to variation in the period of exposure, which was longer in the former (28 days) than the latter (exposure of only 24 hours), as well as first-pass metabolism of polyphenols before it reached the hepatocytes. Additional studies should be employed to investigate other genes, relevant polyphenol conjugates, duration and doses of exposure before primary hepatocytes can be used as a substitute for *in vivo* studies, with reference to xenobiotic metabolism. Differences also exist when using pure polyphenols as compared to enriched extracts containing high levels of the respective polyphenols. However, the change in the expression of *Hsd17β2* by the herbal teas is consistent under the current experimental conditions and primary hepatocytes can be utilised to investigate this aspect in more detail on a protein level.

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CHAPTER 5

GENERAL DISCUSSION

The consumption of the herbal teas rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) is increasing worldwide (Snyman, 2000; De Villiers, 2004) due to the promotion of their health benefits by pharmaceutical and nutraceutical companies. These benefits include anecdotal evidence of alleviation of headaches, fever, asthma, insomnia, colic in infants and skin disorders (Morton, 1983; Joubert and Ferreira, 1996). It has also been shown to have anti-oxidant, anti-mutagenic and anti-cancer properties (Marnewick *et al*., 2000; Standley *et al*., 2001; Richards, 2002). These herbal teas contain a complex mixture of polyphenolic compounds (Rabe*et al*., 1994; Ferreira *et al*., 1995; Ferreira *et al*., 1998; Marias *et al*., 1998; Marais *et al*., 2000; Kamara *et al*., 2003; Kamara *et al*., 2004; Shimamura *et al*., 2006; Iswaldi *et al*., 2011), and have been utilised by the nutraceutical industry to formulate various combinations of polyphenol-enriched extracts in an attempt to explore their health benefits. Most of the biological properties are attributed to the polyphenolic composition of the herbal teas. Studies have shown that polyphenols also interact with xenobiotic metabolising enzymes (Mandlekar *et al*., 2006; Murray, 2006; Rodeiro *et al*., 2008; Sergent *et al*., 2009), causing it to enhance or inhibit the activity of certain pharmaceutical drugs. The potential risk associated with the chronic use of polyphenol-enriched extracts is not clearly understood and needs clarification.

The present study was the first to investigate the effect of polyphenol-enriched extracts of green rooibos and honeybush (*C. genistoides* and *C. subternata*) on the gene expression of a battery (eighty four) of xenobiotic metabolising enzymes in male Fischer 344 rat liver and kidneys *in vivo*. In a subsequent study the modulation of certain phase I and II enzymes was validated in rat primary

hepatocytes utilising the same herbal tea extracts as well as the respective major polyphenolic constituents of rooibos and honeybush, aspalathin and mangiferin. The study was conducted to validate the use of the *in vitro* cell model to predict effects of the herbal tea *in vivo* and to further elucidate the underlying mechanisms regarding changes in gene expression of the xenobiotics metabolising enzymes.

Xenobiotics can undergo three fates upon entering an organism, namely, metabolism by enzymes, spontaneous change into other substances under optimum pH conditions, or be excreted unchanged (Williams, 1972). Following ingestion, xenobiotics pass through the gastrointestinal tract to the liver where the majority of xenobiotic metabolism reactions are carried out by phase I and phase II enzymes in the hepatic cells (Gillette, 1966). The conjugated xenobiotics are then excreted via urinary and/or biliary pathways. In the kidney, certain cells in the proximal convoluted tubuli express immunologically similar xenobiotic metabolising enzymes to hepatocytes (Lock and Reed, 1998; Lash *et al*., 2008) and are therefore capable of actively metabolising many drugs, hormones and other xenobiotics (Anders, 1980; Cummings *et al*., 2000). The conjugated metabolite can either be de-conjugated to its original form or it can be actively reabsorbed into the blood stream (Aleksunes *et al*., 2008; Klaassen and Lu, 2008) by retrograde transporters on the basolateral face of the plasma membrane, such as multidrug resistance-associated proteins. Uptake of chemicals across the basolateral membranes into the kidneys is mediated by secondary active transport systems such as P-glycoprotein, a protein product of the multidrug resistance proteins. The elucidation of renal excretory mechanisms is therefore important in understanding the pharmacologic efficacy and duration of action of xenobiotics (Grantham and

Chonko, 1991; Grundemann *et al*., 1994; Krishna and Klotz, 1994). Functional differences exist between the liver and kidneys with respect to their role in xenobiotic metabolism and excretion.

Quantitative real time polymerase chain reaction (qRT-PCR) and qPCR array analysis showed that the polyphenol-enriched extracts of rooibos and honeybush differentially altered the gene expression of several genes in the liver and kidneys. In the present study, the up-regulation of the glucose phosphate isomerise (*Gpi)* gene by all three herbal teas in the liver indicates that the extracts may increase the energy demand in the cells, therefore the glycolytic gene is switched on to maintain homeostasis regarding ATP production. However, with respect to cancer development, this may enhance cancer cell survival and proliferation thereby promoting cancer progression. Normal cells exhibit metabolic flexibility and are capable of regulating their dependence on glycolysis relative to mitochondrial respiration. This allows cells to cope with the prevailing nutrient availability or energy demands (Gohil *et al*., 2010). Favouring anaerobic glycolytic metabolism appears to be a natural adaptation to reduced oxygen availability (Ramirez *et al*., 2007). However, redirecting energy metabolism toward glycolysis can reduce oxidative damage and suppresses apoptosis (Jeong *et al*., 2004; Hunter *et al*., 2007; Vaughn and Deshmukh, 2008). Many cancer cells therefore rely on glycolysis, which is known as the Warburg effect (Warburg, 1956), whereas normal cells utilise the oxidative phosphorylation in the tricarboxylic acid cycle in the mitochondria for the production of ATP (Kondoh, 2008; Denko, 2008; Ortega *et al*., 2009; Buchakjian and Kornbluth, 2010; Cairns *et al*., 2011). Malignant cells increase their expression of glycolytic enzymes such as *Gpi* and hexokinase 2

(*Hk2*), and glucose uptake which leads to the production of ATP and synthesis of nucleic acids and lipids essential for cell survival and division (Kondoh, 2008; Buchakjian and Kornbluth, 2010; Cairns *et al*., 2011).

The aspalathin-enriched rooibos extract suppressed the expression of the *Abcb1* gene, while the two mangiferin-enriched *Cyclopia* extracts down-regulated the *Hk2* gene in the kidneys. Cancer cells are known to increase the expression of ATP-dependent efflux pumps, the ATP-binding cassette (ABC) transporters including P-glycoprotein (ABCB1), which attributes to its drug resistance (Dean, 2009; Allen *et al*., 2000). Therefore, ABC transporters are widely considered to be one of the most critical targets in the treatment of cancers, especially those with drug resistance. Inhibition of glycolysis is able to inactivate all types of ABC transporters in cancer cells, since their dependence on ATP generation is mainly through enhanced glycolysis. Certain isoforms of enzymes such as HK2 have been reported to be up-regulated and responsible for the enhancement of glycolysis exclusively in cancer cells (Ko *et al*., 2001; Mathupala *et al*., 2009; Wolf *et al*., 2011). It would appear that rooibos and honeybush could selectively alter these pathways and may therefore be used to sensitise the cancer cell's susceptibility to anti-cancer drugs in the kidneys.

The liver and kidneys are equipped with the relevant enzymes to metabolise various forms of xenobiotics including procarcinogens, pharmaceutical drugs and dietary polyphenols. The metabolism of chemical procarcinogenic compounds by the two cytochrome (CYP) P450 enzymes, CYP1A1 and CYP1B1, often lead to the formation of reactive carcinogenic intermediates (Sutter *et al*., 1994; Crofts *et al*.,

1997; Shimada *et al*., 1997; Heidel *et al*., 2000; Shimada *et al*., 2002). CYP1B1 is also over expressed in various cancers, but not in normal hepatic tissues (Murray *et al*., 1997; McFadyen *et al*., 1999), thus studies have reported using the enzyme as a target for anti-cancer drugs (Rochat *et al*., 2001; McFadyen *et al*., 2001). The isoform also biotransforms 17β-estradiol to its 4-hydroxy-metabolites (Hayes *et al*., 1996). CYP1 isoforms are transcriptionally regulated by the aryl hydrocarbon receptor (AhR) (Ramadoss *et al*., 2004) upon activation by AhR ligands such as polyaromatic hydrocarbons. Studies have also shown that polyphenols weakly activate the AhR and can also act as AhR antagonists (Ciolino *et al*., 1999; Casper *et al*., 1999; Quadri *et al*., 2000; Ramadoss *et al*., 2004). The activated AhR translocates to the nucleus and then dissociates from its chaperone proteins and associates with the aryl hydrocarbon nuclear translocator (ARNT) protein. The heterodimer can then bind dioxin response elements (Reyes *et al*., 1992), and regulate the transcription of target genes (CYP1 isoforms). Since the *Arnt* and the *Cyp1b1* genes were up-regulated by *C. subternata*, and the *Cyp1a1* gene by *C. genistoides*, in the kidneys, it would appear that the mangiferin-enriched *Cyclopia* extracts could regulate gene expression of enzymes that bioactivate procarcinogens and therefore favour genotoxic DNA damage and cancer initiation. Conversely another carcinogen-associated cytochrome, *Cyp4b1*, was down-regulated by the rooibos extract in the kidneys, thus may protect against the development of carcinogen-induced DNA lesions. This is supported by the up-regulation of the phase II enzymes, glutathione-S-transferase mu (*Gstm1*) and microsomal Gst (*Mgst1*), and the down-regulation of the glutathione-depletion enzyme, gamma glutathione transferase (*Ggt1*), by rooibos, indicating that the extract could protect the cell against carcinogens when utilising the glutathione

conjugation deactivation pathway. The *C. subternata* extract, also up-regulated the genes of two phase II enzymes, *Gstm4* and *Mgst2*. In contrast the *C. genistoides* extract down-regulated the *Gstm3* and *Gstm5* genes, thereby impairing the clearance of carcinogenic substances by glutathione conjugation.

The expression of genes encoding enzymes that are involved in the metabolism of pharmaceutical drugs and xenobiotics have been differentially altered by *C. subternata* extract in the liver and kidneys. The liver *Cyp2b6* and *Cyp2c13* as well as the kidney esterase 22 (*Es22*) were down-regulated. *Cyp2e1*, carboxyl esterases (*Ces*) 1 and 2 were up-regulated in the liver while the expression of arachidonate 15-lipoxygenase (*Alox15*) and fatty acid amide hydrolase (*Faah*) were up-regulated in the kidney. These findings indicate that the extract may selectively enhance or inhibit the phase I metabolism of drugs and other xenobiotics. It should also be noted that the *C. genistoides* and rooibos extracts did not alter the gene expression of phase I enzymes and may therefore not transcriptionally interfere with phase I metabolism.

All three herbal tea extracts are likely to affect alcohol metabolism in the liver. The alcohol dehydrogenase (*Adh4*) gene, which codes for the enzyme responsible for the conversion of alcohol to the toxic acetaldehyde, was up-regulated by *C. genistoides*. Aldehyde dehydrogenase (*Ald1a1*), responsible for the catalysis of acetaldehyde to its less toxic form acetate, was up-regulated by all three extracts, indicating that concurrent ingestion of these extracts and alcohol could be beneficial in the prevention of the “hang-over” effect observed after excessive alcohol consumption. This is in agreement with studies indicating that extracts of

green tea (*Camellia sinensis*) promote alcohol metabolism (Skrzydlewska *et al*., 2002).

Polyphenols have been shown to play a role in steroid metabolism (Yang *et al*., 2001). In the current study, *Cyp19a1*, which codes the aromatase enzyme responsible for the conversion of testosterone to estradiol and androstenedione to estrone, was up-regulated by the rooibos and *C. genistoides* extracts in the liver and kidneys, respectively. However, it does not coincide with the study by Wang *et al*. (1994), which demonstrated an inhibitory effect on aromatase by flavonoids and suggested that flavonoid-rich foods may contribute to the reduction of estrogen-dependent disease, such as breast cancer. The specific flavonoids, however, may play a role. The herbal tea extracts also regulated the gene expression of three isoforms of the 17-beta hydroxysteroid dehydrogenases, *Hsd17β1*, *2* and *3*, the former two was down-regulated by the extracts in the liver, whereas the latter was up-regulated by *C. genistoides* in the kidneys. The liver and kidneys are known to be the major organs involved in steroid metabolism and clearance, especially in the degradation thereby regulating their levels in the blood (West and Samuels, 1951; Kley *et al*., 1979). The 17-beta hydroxysteroid dehydrogenases mainly catalyses the steps of sex steroid degradation by converting biologically active forms to inactive metabolites (Wu *et al*., 1993; Casey *et al*., 1994). The herbal tea extracts seem to disrupt this balance by the impairment of estradiol and testosterone breakdown in the liver and kidneys. Recent studies indicated that *C. subternata and C. genistoides* exhibited phytoestrogen activity in breast cancer cells (Mfenyana *et al*., 2008). This would imply that these phytoestrogens will be antagonists for the estrogen receptor presumably resulting in estradiol accumulation. However, the

present study suggests the impairment of estrogen degradation by the mangiferin-enriched honeybush extracts which tend to contradict the phytoestrogenic properties of honeybush shown under *in vitro* conditions. It would appear that differences exist when considering the effect of the herbal teas on steroid metabolism under *in vivo* conditions which do not necessarily reflect *in vitro* conditions. This is also in agreement with the current study where the expression of specific xenobiotic metabolising enzymes by the herbal tea extracts differs under *in vivo* and *in vitro* conditions.

Of interest is the up-regulation of the myeloperoxidase gene (18.60 fold) by *C. subternata* extract in the kidneys. The enzyme is involved in inflammatory responses which can be beneficial in terms of infection (Klebanoff, 2005), but harmful when leached out of the cell, where it can cause cellular damage (Malle *et al*., 2007) as in the case of pyelonephritis and glomerular nephritis (Odobasic *et al*., 2007). The herbal tea extract may therefore expose the kidney cells to reactive oxidants that can be unfavourable.

Modulation of the expression of the xenobiotic metabolising enzyme observed could be attributed to differences in the polyphenolic content of the aspalathin-enriched rooibos extract and mangiferin-enriched extracts of *C. genistoides* and *C. subternata*. However, of the honeybush herbal teas, the *C. subternata* extract demonstrated a higher response regarding the modulation of the expression of different xenobiotic metabolism enzymes in the liver and kidneys, despite it having lower mangiferin content. This indicates that other polyphenols in the extract and/or non-polyphenolic constituents may have caused the effects observed. Of note is

the high eriocitrin (1.1 g/100 g extract) and scolymoside (1.29 g/100 g extract) contents of *C. subternata* extract which were absent in the *C. genistoides* extract. The hesperidin and eriodictyol-glucoside contents were also slightly higher in the *C. subternata* extract. It should also be noted that the total polyphenol content of the *C. subternata* extract as well as the total polyphenol intake of the rats was significantly higher when compared to the *C. genistoides* treated rats. This could also be related to the increase of the serum parameter total bilirubin which suggest that the biliary system was adversely affected by *C. subternata*.

Differences were observed between liver and kidney in terms of the effect of the herbal tea extracts on gene expression. The genes affected in the liver and kidneys were aligned with their main function. In the liver the genes involved in alcohol, energy, steroid and phase I metabolism were altered. The lack of changes to the phase II genes in the liver could be ascribed to its high capacity to metabolise polyphenols which masked their adverse effects. In the kidneys, however mostly phase II genes, transporters and only certain phase I genes were altered. This is due to the kidney being the main organ for excretion although it retains capacity to metabolise xenobiotics, which is far less effective when compared to the liver.

Since the herbal tea extracts displayed differential gene expression in the liver, a primary hepatocyte model was used to validate changes to selected genes *in vitro*. The results indicated variable effects regarding the expression of the selected genes. It seems that rat primary hepatocytes can be used to predict *in vivo* effects of the herbal tea extracts on *Hsd17β2,* however, care should be taken when considering effects on *Cyp2e1* and *Comt.* In this particular study, the polyphenols,

aspalathin and mangiferin, were also included to investigate the differences between complex mixtures and single compounds. Aspalathin up-regulated the *Comt* gene and down-regulated the *Hsd17β2* gene, whereas the aspalathin-enriched rooibos extract did not significantly alter the genes. Mangiferin also differed from the mangiferin-enriched *Cyclopia* extracts by significantly down-regulating both genes. It is known that complex mixtures versus single compounds do have varying effects when monitoring a specific biological event in a cell. Active transport of a food borne carcinogen into Caco-2 mono layers was less effective in the presence of a single flavonoid as compared to the effect of a combination of flavonoids (Schutte *et al*., 2008). In contrast, when utilising a rat uterothropic assay a mixture of estrogens showed an intermediate or reduced effect compared to a moderate stimulating effect by a single estrogen (Tinwell and Ashby, 2004). Of interest is that the expression of the *Hsd17β2* and *Comt* genes was significantly reduced by aspalathin and mangiferin while the enriched extracts showed an intermediate effect. It would appear that the gene modulating effects of a single polyphenol may be masked in the presence of complex mixtures containing other polyphenolic and non-polyphenolic constituents such as the polyphenol-enriched herbal tea extracts utilised in the present study.

In conclusion, although human intervention studies are yet to be conducted with regards to the polyphenol-enriched extracts of rooibos and honeybush herbal teas, the present findings showed that the genes of xenobiotic metabolising enzymes are differentially altered by the herbal teas. Caution should therefore be taken when using the herbal tea extracts in combination with pharmaceutical drugs and/or xenobiotics and a moderate consumption is advised. Future studies will therefore

include the effects of the herbal tea extracts, their polyphenols and metabolites on enzyme interactions in terms of xenobiotic metabolism.

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ADDENDUM I

Table 1 Commonly prescribed cytochrome P450 inducers (Sandson, 2004).

1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4, 5, 7
broccoli brussel sprouts char-grilled meat insulin methylcholanthrene modafinil nafcillin beta-naphthoflavone omeprazole tobacco	phenobarbital rifampin	rifampin	rifampin secobarbital	carbamazepine norethindrone NOT pentobarbital prednisone rifampin	dexamethasone rifampin	ethanol isoniazid	HIV Antivirals: efavirenz nevirapine barbiturates carbamazepine efavirenz glucocorticoids modafinil nevirapine oxcarbazepine phenobarbital phenytoin pioglitazone rifabutin rifampin St. John's wort troglitazone

Table 2 Commonly prescribed cytochrome P450 inhibitors (Sandson, 2004).

1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4, 5, 7
fluvoxamine ciprofloxacin cimetidine amiodarone fluoroquinolones furafylline interferon methoxsalen mibefradil	thiotepa ticlopidine	gemfibrozil trimethoprim glitazones montelukast quercetin	fluconazole amiodarone fenofibrate fluvastatin fluvoxamine isoniazid lovastatin phenylbutazone probenicid sertraline sulfamethoxazole sulfaphenazole teniposide voriconazole zafirlukast	PPIs: lansoprazole omeprazole pantoprazole rabeprazole chloramphenicol cimetidine felbamate fluoxetine fluvoxamine indomethacin ketoconazole modafinil oxcarbazepine probenicid ticlopidine topiramate		diethyldithiocarbamate disulfiram	HIV Antivirals: indinavir nelfinavir ritonavir clarithromycin itraconazole ketoconazole nefazodone saquinavir telithromycin aprepitant erythromycin fluconazole grapefruit juice verapamil diltiazem cimetidine amiodarone NOT azithromycin chloramphenicol ciprofloxacin delaviridine diethyldithiocarbamate fluvoxamine gestodene imatinib mibefradil mifepristone norfloxacin norfluoxetine star fruit voriconazole

Table Substrates of the major isoforms of cytochrome P450 (Sandson, 2004).

1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4, 5, 7
amitriptyline caffeine clomipramine clozapine cyclobenzaprine estradiol fluvoxamine haloperidol imioramine N-DeMe mexilletine naproxen olanzapine ondansetron phenacetin_ acetaminophen NAPQI propranolol riluzole ropivacaine tacrine theophylline tizanidine verapamil (R)warfarin Zileuton zolmitriptan	bupropion cyclophosphamide efavirenz ifosfamide methadone	paclitaxel torsemide amodiaquine cerivastatin repaglinide	NSAIDs: diclofenac ibuprofen lornoxicam meloxicam S-naproxen –Nor piroxicam Suprofen Oral Hypoglycemic Agents: tolbutamide glipizide Angiotensin II Blockers: losartan irbesartan Sulfonylureas: glyburide glibenclamide glipizide glimepiride tolbutamide amitriptyline celecoxib fluoxetine fluvastatin glyburide nateglinide phenytoin-4-OH2 rosiglitazone tamoxifen torsemide S-warfarin	Proton Pump Inhibitors: lansoprazole omeprazole pantoprazole rabeprazole Anti-epileptics: diazepam_Nor phenytoin(O) S-mephenytoin phenobarbitone amitriptyline carisoprodol citalopram chloramphenicol clomipramine cyclophosphamide hexobarbital imipramine N-DeME indomethacin R-mephobarbital moclobemide nelfinavir nilutamide primidone progesterone proguanil propranolol teniposide R-warfarin_8-OH		Anesthetics: enflurane halothane isoflurane methoxyflurane sevoflurane acetaminophen_ NAPQI aniline2 benzene chlorzoxazone ethanol N,N-dimethyl formamide theophylline_ 8-OH	Macrolide antibiotics: clarithromycin erythromycin (not 3A5) NOT azithromycin telithromycin Anti-arrhythmics: quinidine_3OH (not 3A5) Benzodiazepines: alprazolam diazepam_3OH midazolam triazolam Immune Modulators: cyclosporine tacrolimus (FK506) HIV Antivirals: indinavir nelfinavir ritonavir saquinavir Prokinetic: cisapride Antihistamines: astemizole chlorpheniramine terfenadine Calcium Channel Blockers: amlodipine diltiazem

							felodipine lercanidipine nifedipine2 nisoldipine nitrendipine verapamil HMG CoA Reductase Inhibitors: atorvastatin cerivastatin lovastatin NOT pravastatin simvastatin Steroid 6beta-OH: estradiol hydrocortisone progesterone testosterone Miscellaneous: alfentanyl aprepitant aripiprazole buspirone cafergot caffeine_TMU cilostazol cocaine codeine- Ndemethylation dapsone dexamethasone dextromethorphan docetaxel domperidone eplerenone fentanyl

							finasteride gleevec haloperidol irinotecan LAAM lidocaine methadone nateglinide ondansetron pimozide propranolol quetiapine quinine risperidone NOT rosuvastatin salmeterol sildenafil sirolimus tamoxifen taxol terfenadine trazodone vincristine zaleplon ziprasidone zolpidem

ADDENDUM II

Table 1 The feed intake of individual animals calculated as g feed/100 g bodyweight of male Fisher rats fed with aspalathin-enriched rooibos and mangiferin-enriched honeybush extracts for a period of 30 days.

Animal No.	Control (Neg) g feed/100g BW	Rooibos g feed/100g BW	*C. genistoides* g feed/100g BW	*C. subternata* g feed/100g BW
1	8.93	9.80	9.97	9.91
2	10.38	10.36	9.55	10.52
3	9.95	9.60	9.63	10.32
4	9.60	9.60	10.11	10.03
5	9.69	9.07	10.89	10.15
6	9.87	9.42	10.10	9.83
7	9.94	9.49	10.18	10.71
8	9.91	10.21		11.35
9	9.17	9.50	9.14	10.49
10	9.87	9.39	10.18	10.24
AVE	**9.73**	9.65	9.97	10.35
STD	**1.66**	1.42	1.56	1.66

** The concentrations of the extracts were 2.0 g rooibos extract/kg diet and 2.5 g honeybush extract /kg diet.*

Table 2 Individual bodyweight gain and relative liver and kidney weight of male Fisher rats fed with aspalathin-enriched rooibos and mangiferin-enriched honeybush extracts for a period of 30 days.

Group	No.	Bodyweight gain (g)	Relative liver weight (%)	Relative kidney weight (%)
1. Control (Neg)	1	87	3.75	0.68
	2	100	3.25	0.62
	3	78	3.44	0.64
	4	87	3.60	0.64
	5	103	3.62	0.70
	6	90	3.60	0.67
	7	87	3.45	0.63
	8	97	3.46	0.61
	9	109	3.60	0.63
	10	107	3.90	0.64
AVE		**94.50**	**3.57**	**0.65**
STD		**10.20**	**0.18**	**0.03**
2. Rooibos	1	69	3.74	0.62
	2	75	3.68	0.65
	3	93	3.81	0.59
	4	105	3.99	0.62
	5	80	3.57	0.63
	6	95	3.94	0.67
	7	95	3.42	0.62
	8	107	3.67	0.59
	9	79	3.61	0.62
	10	97	3.57	0.68
AVE		**89.50**	**3.70**	**0.63**
STD		**12.94**	**0.18**	**0.03**
3. C. genistoides	1	102	3.88	0.66
	2	96	4.18	0.65
	3	101	3.65	0.64
	4	91	3.76	0.70
	5	88	3.55	0.66
	6	99	3.70	0.63
	7	90	3.71	0.64
	9	70	3.60	0.70
	10	91	4.26	0.70
AVE		**92.00**	**3.81**	**0.67**
STD		**9.70**	**0.25**	**0.03**
4. C. subternata	1	86	3.36	0.63
	2	110	3.65	0.65
	3	104	3.42	0.62
	4	87	3.32	0.71
	5	78	3.52	0.62
	6	95	3.74	0.69
	7	80	3.52	0.64
	8	108	3.96	0.70
	9	80	3.66	0.64
	10	107	3.56	0.68
AVE		**93.50**	**3.57**	**0.66**
STD		**12.83**	**0.19**	**0.03**

Table 3 GeneBank codes of genes encoding xenobiotic metabolising enzymes and transporters.

Gene Table

Position	Unigene	GeneBank	*Symbol*	Description	Gene Name
A01	Rn.144554	NM_012623	*Abcb1b*	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	Abcb1/Mdr1
A02	Rn.154810	NM_133401	*Abcb1a*	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	Mdr1a
A03	N/A	NM_012690	*Abcb4*	ATP-binding cassette, sub-family B (MDR/TAP), member 4	Mdr2/Pgy3
A04	Rn.10495	NM_022281	*Abcc1*	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	Abcc1a/Avcc1a
A05	Rn.54493	NM_022935	*Abp1*	Amiloride binding protein 1 (amine oxidase, copper-containing)	Abp
A06	Rn.40222	NM_019286	*Adh1*	Alcohol dehydrogenase 1 (class I)	Adh/Adh1a
A07	Rn.98159	NM_017270	*Adh4*	Alcohol dehydrogenase 4 (class II), pi polypeptide	ADH-1/Ac1002
A08	Rn.91370	NM_013149	*Ahr*	Aryl hydrocarbon receptor	Ahr
A09	Rn.3941	NM_012899	*Alad*	Aminolevulinate, delta-, dehydratase	ALADR/aminolevulinatedelta-dehydratase
A10	Rn.6132	NM_022407	*Aldh1a1*	Aldehyde dehydrogenase family 1, member A1	AHD2/Aldh1
A11	Rn.11318	NM_031010	*Alox15*	Arachidonate 15-lipoxygenase	12-LOX/Alox12
A12	Rn.9662	NM_012822	*Alox5*	Arachidonate 5-lipoxygenase	LOX5A
B01	Rn.32351	NM_138828	*Apoe*	Apolipoprotein E	APOEA
B02	Rn.10520	NM_012780	*Arnt*	Aryl hydrocarbon receptor nuclear translocator	Arnt1
B03	Rn.7354	XM_213848	*Asna1*	ArsA arsenite transporter, ATP-binding, homolog 1 (bacterial)	N/A
B04	Rn.9865	NM_053850	*Blvra*	Biliverdin reductase A	Blvra
B05	Rn.161812	XM_214823	*Blvrb_predicted*	Biliverdin reductase B (flavin reductase (NADPH)) (predicted)	LOC292737
B06	Rn.161717	NM_031565	*Ces1*	Carboxylesterase 1	MGC156521
B07	Rn.14535	NM_133586	*Ces2*	Carboxylesterase 2 (intestine, liver)	CES RL4/rCES2
B08	Rn.16695	NM_001011955	*Chst1*	Carbohydrate (keratan sulfate Gal-6)	LOC295934

				sulfotransferase 1	
B09	Rn.220	NM_012531	*Comt*	Catechol-O-methyltransferase	Comt
B10	Rn.35994	NM_138877	*Cyb5r3*	Cytochrome b5 reductase 3	Dia1/Nadhcb5
B11	Rn.10172	NM_012753	*Cyp17a1*	Cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17
B12	Rn.21402	NM_017085	*Cyp19a1*	Cytochrome P450, family 19, subfamily a, polypeptide 1	Aromatase/Cyp19
C01	Rn.10352	NM_012540	*Cyp1a1*	Cytochrome P450, family 1, subfamily a, polypeptide 1	AHH/AHRR
C02	Rn.5563	NM_012541	*Cyp1a2*	Cytochrome P450, family 1, subfamily a, polypeptide 2	CYPD45/P-450d
C03	Rn.10125	NM_012940	*Cyp1b1*	Cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1
C04	Rn.10847	NM_053763	*Cyp27b1*	Cytochrome P450, family 27, subfamily b, polypeptide 1	Cyp40
C05	Rn.144570	NM_017156	*Cyp2b15*	Cytochrome P450, family 2, subfamily b, polypeptide 15	Cyp2b15
C06	Rn.127147	M19973	*Cyp2b6*	Cytochrome P450, subfamily IIB (phenobarbital-inducible), polypeptide 6	Cyp2b6
C07	Rn.82715	NM_138514	*Cyp2c13*	Cytochrome P450 2c13	Cyp2c38
C08	Rn.91122	XM_001066767	*Cyp2c6_predicted*	Cytochrome P450, subfamily IIC6 (predicted)	Cyp2c6/PB1
C09	Rn.1247	NM_017158	*Cyp2c7*	Cytochrome P450, family 2, subfamily c, polypeptide 7	Cyp2c39
C10	Rn.1372	NM_031543	*Cyp2e1*	Cytochrome P450, family 2, subfamily e, polypeptide 1	Cyp2e
C11	Rn.91120	NM_013105	*Cyp3a23/3a1*	Cytochrome P450, family 3, subfamily a, polypeptide 23/polypeptide 1	CYP/Cyp3a1
C12	Rn.86651	NM_016999	*Cyp4b1*	Cytochrome P450, family 4, subfamily b, polypeptide 1	Cyp4b1
D01	Rn.3603	NM_012844	*Ephx1*	Epoxide hydrolase 1, microsomal	MEH8
D02	Rn.89119	NM_024132	*Faah*	Fatty acid amide hydrolase	Faah
D03	Rn.33703	NM_012558	*Fbp1*	Fructose-1,6-biphosphatase 1	Fdp

D04	Rn.91245	NM_017007	*Gad1*	Glutamic acid decarboxylase 1	Gad67
D05	Rn.29951	NM_012563	*Gad2*	Glutamic acid decarboxylase 2	gad65
D06	Rn.7863	NM_013120	*Gckr*	Glucokinase regulatory protein	GLRE
D07	Rn.10010	NM_053840	*Ggt1*	Gamma-glutamyltransferase 1	GGLUT/Ggt
D08	Rn.84435	NM_207592	*Gpi*	Glucose phosphate isomerase	Amf/Gpi1
D09	Rn.11323	NM_030826	*Gpx1*	Glutathione peroxidase 1	GSHPx/GSHPx-1
D10	Rn.3503	NM_183403	*Gpx2*	Glutathione peroxidase 2	GPX-GI/GSHPx-2
D11	Rn.108074	NM_022525	*Gpx3*	Glutathione peroxidase 3	GSHPx-3/GSHPx-P
D12	Rn.3647	NM_017165	*Gpx4*	Glutathione peroxidase 4	Phgpx/gpx-4
E01	Rn.218434	XM_001059839	*Gpx5*	Glutathione peroxidase 5	Gpx5
E02	Rn.19721	NM_053906	*Gsr*	Glutathione reductase	Gsr
E03	Rn.10460	NM_031509	*Gsta3*	Glutathione S-transferase A3	Gsta1/Gsta5
E04	Rn.57528	XM_217195	*Gsta4*	Glutathione S-transferase, alpha 4	LOC300850
E05	Rn.202944	NM_017014	*Gstm1*	Glutathione S-transferase, mu 1	GSTA3
E06	Rn.625	NM_177426	*Gstm2*	Glutathione S-transferase, mu 2	GSTA4
E07	Rn.6036	NM_031154	*Gstm3*	Glutathione S-transferase, mu type 3	Gstm3
E08	Rn.209043	NM_020540	*Gstm4*	Glutathione S-transferase M4	GstYb4
E09	Rn.9158	NM_172038	*Gstm5*	Glutathione S-transferase, mu 5	Gstm5
E10	Rn.87063	NM_138974	*Gstp1*	Glutathione-S-transferase, pi 1	GST-P/Gst3
E11	Rn.11122	NM_053293	*Gstt1*	Glutathione S-transferase theta 1	GSTYRS
E12	Rn.91375	NM_012735	*Hk2*	Hexokinase 2	Hk2
F01	Rn.10594	NM_012851	*Hsd17b1*	Hydroxysteroid (17-beta) dehydrogenase 1	17BHD1
F02	Rn.10515	NM_024391	*Hsd17b2*	Hydroxysteroid (17-beta) dehydrogenase 2	Hsd17b2
F03	Rn.10895	NM_054007	*Hsd17b3*	Hydroxysteroid (17-beta) dehydrogenase 3	Hsd17b3
F04	Rn.60583	XM_220831	*Lpo_predicted*	Lactoperoxidase (predicted)	N/A
F05	Rn.9560	XM_215403	*Marcks*	Myristoylated alanine rich protein kinase C substrate	KINC/Macs

F06	Rn.2580	NM_134349	*Mgst1*	Microsomal glutathione S-transferase 1	MGC72699
F07	Rn.7854	XM_215562	*Mgst2_predicted*	Microsomal glutathione S-transferase 2 (predicted)	LOC295037
F08	Rn.1916	XM_213943	*Mgst3_predicted*	Microsomal glutathione S-transferase 3 (predicted)	LOC289197
F09	Rn.47782	XM_220830	*Mpo*	Myeloperoxidase	Mpo
F10	Rn.11325	NM_053968	*Mt3*	Metallothionein 3	GIF/Mt-3
F11	Rn.10494	XM_342975	*Mthfr_predicted*	5,10-methylenetetrahydrofolate reductase (NADPH) (predicted)	Mthfr
F12	Rn.37420	NM_001037315	*Nat1*	N-acetyltransferase 1 (arylamine N-acetyltransferase)	Nat2
G01	Rn.10400	NM_012611	*Nos2*	Nitric oxide synthase 2, inducible	Nos2a/iNos
G02	Rn.44265	NM_021838	*Nos3*	Nitric oxide synthase 3, endothelial cell	eNos
G03	Rn.11234	NM_017000	*Nqo1*	NAD(P)H dehydrogenase, quinone 1	Dia4
G04	Rn.48821	NM_012624	*Pklr*	Pyruvate kinase, liver and red blood cell	PK1/PKL
G05	Rn.1556	NM_053297	*Pkm2*	Pyruvate kinase, muscle	PKM12/Pk3
G06	Rn.20732	NM_032077	*Pon1*	Paraoxonase 1	Pon1
G07	Rn.1100	NM_001013082	*Pon2*	Paraoxonase 2	N/A
G08	Rn.16469	NM_001004086	*Pon3*	Paraoxonase 3	MGC95026
G09	Rn.34679	XM_237241	*Smarcal1_predicted*	Swi/SNF related matrix associated, actin dependent regulator of chromatin, subfamily a-like 1 (predicted)	N/A
G10	Rn.6147	NM_001034083	*Snn*	Stannin	Snn
G11	Rn.4620	NM_017070	*Srd5a1*	Steroid 5 alpha-reductase 1	MGC156498
G12	Rn.202951	NM_017154	*Xdh*	Xanthine dehydrogenase	XOR
H01	Rn.973	NM_001007604	*Rplp1*	Ribosomal protein, large, P1	MGC72935
H02	Rn.47	NM_012583	*Hprt*	Hypoxanthine guanine phosphoribosyl transferase	Hgprtase/Hprt1
H03	Rn.92211	NM_173340	*Rpl13a*	Ribosomal protein L13A	Rpl13a
H04	Rn.107896	NM_017025	*Ldha*	Lactate dehydrogenase A	Ldh1
H05	Rn.94978	NM_031144	*Actb*	Actin, beta	Actx
H06	N/A	U26919	RGDC	Rat Genomic DNA Contamination	RGDC
H07	N/A	SA_00104	RTC	Reverse Transcription	RTC

				Control	
H08	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H09	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H10	N/A	SA_00103	PPC	Positive PCR Control	PPC
H11	N/A	SA_00103	PPC	Positive PCR Control	PPC
H12	N/A	SA_00103	PPC	Positive PCR Control	PPC

Table 4 Ratios indicating the integrity of the RNA isolated from liver and kidney tissue after exposure to polyphenol-enriched herbal tea extracts.

SAMPLE	***LIVER***		***KIDNEY***	
	260/280a	***260/230b***	***260/280a***	***260/230b***
C1	1.99	1.76	2.00	1.60
C4	1.99	1.60	2.00	1.70
C9	1.97	1.40	2.10	1.80
R7	1.98	1.57	2.10	1.40
R8	1.98	1.54	2.10	1.80
R9	1.98	1.51	2.00	1.20
S7	2.00	1.43	2.10	1.60
S8	1.98	1.49	2.10	1.40
S9	2.00	1.49	2.10	1.30
G5	1.98	1.68	2.10	1.70
G7	1.99	1.52	2.10	1.60
G9	1.99	1.39	2.10	1.60

[a] Indicates protein and/or DNA contamination (Where ≥2 indicates pure RNA) [b]Indicates salt contamination (Where ≥1.5 indicates pure RNA). C= Control group; R= Aspalathin-enriched rooibos; S= Mangiferin- enriched C. subternata; G= Mangiferin- enriched C. genistoides.

Table 5 Efficiency of the reverse transcription reaction and the genomic DNA contamination controls of treated and untreated liver samples.

	Control/untreated			***Rooibos***			***C. subternata***			***C. genistoides***		
Replicates	1	2	3	1	2	3	1	2	3	1	2	3
ΔCt (Ave RTC-Ave PPC)	0.61	0.63	1.06	1.06	1.30	1.21	1.04	0.96	0.64	0.81	1.36	0.53
RT efficiency	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
	Control/untreated			***Rooibos***			***C. subternata***			***C. genistoides***		
Replicates	1	2	3	1	2	3	1	2	3	1	2	3
C_t (GDC)	35	35	35	35	35	32.74	35	34.41	33.46	35	35	35
Genomic DNA	Pass	Pass	Pass	Pass	Pass	Fail	Pass	Fail	Fail	Pass	Pass	Pass

Table 6 Gene profile of xenobiotic metabolising enzymes in the liver after exposure to enriched extracts of rooibos and honeybush herbal teas.

Gene symbol	p value	Test /Control	p value	Test /Control	p value	Test /Control
	Rooibos		*C. genistoides*		*C. subternata*	
Abcb1b	0.860	-1.073	0.095	1.834	0.138	1.551
Abcb1a	0.112	-1.345	0.697	-1.084	0.161	-1.272
Abcb4	0.177	-1.495	0.333	-1.355	0.253	-1.361
Abcc1	0.535	-1.264	0.376	-1.406	0.674	-1.178
Abp1	0.637	1.766	0.600	1.674	0.500	2.175
Adh1	0.673	-1.152	0.331	1.337	0.610	1.088
Adh4	0.286	1.232	0.043	1.333	0.081	1.409
Ahr	0.523	-1.451	0.680	-1.283	0.551	-1.417
Alad	0.581	1.047	0.294	1.127	0.668	1.064
Aldh1a1	0.049	2.393	0.028	1.673	0.028	2.472
Alox15	0.098	-1.227	0.151	-1.750	0.052	-1.551
Alox5	0.643	-1.915	0.493	-2.132	0.650	-1.895
Apoe	0.342	1.166	0.742	-1.040	0.947	1.016
Arnt	0.520	-1.074	0.131	1.347	0.833	1.031
Asna1	0.660	1.143	0.864	1.041	0.927	1.022
Blvra	0.184	1.312	0.539	1.141	0.669	1.112
Blvrb_predicted	0.291	-2.399	0.342	-2.188	0.350	-2.144
Ces1	0.083	1.287	0.089	1.262	0.040	1.319
Ces2	0.308	1.267	0.070	1.392	0.002	1.606
Chst1	0.808	1.083	0.152	1.832	0.869	1.075
Comt	0.783	1.051	0.356	1.314	0.645	1.129
Cyb5r3	0.253	1.179	0.054	1.499	0.362	1.135
Cyp17a1	0.247	-1.390	0.959	1.017	0.345	1.296
Cyp19a1	0.027	1.980	0.337	-1.298	0.874	1.102
Cyp1a1	0.790	-1.114	0.919	1.036	0.934	-1.046
Cyp1a2	0.929	-1.010	0.213	1.246	0.455	1.130
Cyp1b1	0.732	-1.242	0.520	-1.372	0.626	1.695
Cyp27b1	0.458	-2.032	0.490	-1.804	0.726	-1.351
Cyp2b15	0.264	2.145	0.608	-1.508	0.809	-1.239
Cyp2b6	0.162	-1.418	0.824	-1.102	0.033	-1.287
Cyp2c13	0.120	-1.394	0.753	-1.087	0.035	-1.334
Cyp2c6_predicted	0.352	1.198	0.099	1.236	0.204	1.182
Cyp2c7	0.142	1.247	0.680	1.114	0.235	1.298
Cyp2e1	0.112	1.265	0.065	1.279	0.034	1.230
Cyp3a23/3a1	0.523	1.272	0.418	1.324	0.566	1.204
Cyp4b1	0.104	-1.972	0.662	1.107	0.484	-1.356
Ephx1	0.792	1.040	0.496	-1.138	0.438	1.121
Faah	0.906	-1.051	0.567	-1.270	0.501	-1.382
Fbp1	0.338	1.067	0.337	1.160	0.587	1.046
Gad1	0.213	-3.134	0.703	-1.125	0.555	-1.182
Gad2	0.947	1.034	0.709	1.268	0.575	1.460

Gene symbol	p value	Test /Control	p value	Test /Control	p value	Test /Control
	Rooibos		*C. genistoides*		*C. subternata*	
Gckr	0.492	-1.511	0.838	-1.120	0.371	-1.578
Ggt1	0.480	-1.682	0.769	-1.193	0.474	-1.780
Gpi	0.007	2.475	0.005	2.175	0.013	1.919
Gpx1	0.840	1.053	0.729	-1.086	0.536	1.130
Gpx2	0.700	1.179	0.270	-1.575	0.486	-1.317
Gpx3	0.141	1.377	0.453	1.149	0.880	-1.050
Gpx4	0.163	1.131	0.256	1.121	0.597	1.057
Gpx5	0.964	1.038	0.880	-1.055	0.722	-1.189
Gsr	0.120	1.269	0.691	1.051	0.224	1.201
Gsta3	0.196	1.462	0.256	1.377	0.196	1.427
Gsta4	0.320	-1.300	0.965	1.013	0.349	-1.413
Gstm1	0.940	1.020	0.956	1.013	0.946	-1.018
Gstm2	0.296	1.313	0.564	1.156	0.776	1.100
Gstm3	0.557	1.079	0.129	1.298	0.926	-1.016
Gstm4	0.715	-1.054	0.065	-1.217	0.692	-1.070
Gstm5	0.705	-1.206	0.843	-1.103	0.761	-1.152
Gstp1	0.605	-1.155	0.562	1.160	0.374	-1.370
Gstt1	0.863	-1.036	0.940	-1.016	0.992	-1.002
Hk2	0.922	1.042	0.167	-2.088	0.285	-1.264
Hsd17b1	0.157	-1.814	0.041	-1.716	0.173	-1.781
Hsd17b2	0.008	-2.416	0.014	-3.410	0.042	-2.085
Hsd17b3	0.972	1.027	0.713	1.204	0.496	-1.593
Lpo_predicted	0.584	-1.837	0.948	-1.076	0.602	-1.871
Marcks	0.596	-1.194	0.640	-1.144	0.261	-1.183
Mgst1	0.101	1.153	0.321	1.136	0.459	1.105
Mgst2_predicted	0.210	-1.405	0.724	-1.090	0.197	-1.383
Mgst3_predicted	0.755	1.026	0.285	1.123	0.847	1.009
Mpo	0.109	-1.818	0.511	1.169	0.247	-1.897
Mt3	0.299	2.281	0.443	1.740	0.239	2.624
Mthfr_predicted	0.408	-1.331	0.089	-1.349	0.234	-1.232
Nat1	0.508	-1.426	0.910	1.049	0.858	-1.104
Nos2	0.928	1.078	0.924	1.070	0.315	-2.977
Nos3	0.113	-1.546	0.569	1.420	0.186	-1.526
Nqo1	0.239	1.269	0.112	1.616	0.246	3.619
Pklr	0.700	-1.077	0.242	1.340	0.882	1.027
Pkm2	0.468	1.172	0.071	1.518	0.274	1.300
Pon1	0.808	1.058	0.320	1.351	0.362	1.214
Pon2	0.943	1.012	0.644	-1.079	0.693	-1.097
Pon3	0.931	1.019	0.958	1.009	0.479	1.135
Smarcal1_predicted	0.072	-1.655	0.418	-1.268	0.712	-1.093
Snn	0.233	-9.741	0.762	1.051	0.703	-1.107
Srd5a1	0.964	-1.020	0.737	1.155	0.672	1.156
Xdh	0.500	-1.130	0.990	1.002	0.338	1.123

Gene symbol	p value	Test /Control	p value	Test /Control	p value	Test /Control
	Rooibos		*C. genistoides*		*C. subternata*	
Rplp1	0.539	-1.051	0.541	1.073	0.549	1.078
Hprt	0.981	-1.003	0.154	1.299	0.419	1.132
Rpl13a	0.324	-1.243	0.251	-1.508	0.321	-1.319
Ldha	0.753	1.073	0.938	-1.020	0.782	-1.061
Actb	0.085	1.222	0.550	1.103	0.170	1.146

Table 7 Gene profile of xenobiotic metabolising enzymes and transporters in the kidneys after exposure to enriched extracts of rooibos and honeybush herbal teas.

Gene symbol	p value	Test /Control	p value	Test /Control	p value	Test /Control
	Rooibos		*C. genistoides*		*C. subternata*	
Abcb1b	0.735	-1.069	0.645	-1.100	0.239	-1.401
Abcb1a	0.007	-1.851	0.580	-1.315	0.205	-1.889
Abcb4	0.293	-1.665	0.869	1.066	0.584	-1.170
Abcc1	0.159	1.318	0.809	1.108	0.268	1.393
Abp1	0.971	-1.074	0.311	5.299	0.419	3.515
Adh1	0.407	-1.361	0.549	1.325	0.400	1.424
Adh4	0.790	1.222	0.538	1.529	0.461	1.667
Ahr	0.370	1.168	0.967	-1.011	0.683	1.125
Alad	0.626	1.060	0.997	-1.001	0.139	1.291
Aldh1a1	0.668	1.075	0.550	-1.081	0.856	1.043
Alox15	0.227	1.439	0.974	1.010	0.049	1.710
Alox5	0.749	-1.527	0.749	1.562	0.717	1.428
Apoe	0.954	-1.025	0.814	1.127	0.972	-1.021
Arnt	0.661	1.066	0.967	-1.010	0.030	1.303
Asna1	0.339	-1.192	0.906	-1.027	0.901	1.032
Blvra	0.837	1.027	0.418	-1.220	0.156	-1.397
Blvrb_predicted	0.751	-1.052	0.691	-1.099	0.965	-1.005
Ces1	0.792	-1.042	0.846	-1.084	0.045	-1.230
Ces2	0.167	-1.238	0.501	-1.147	0.084	-1.297
Chst1	0.339	1.296	0.755	1.082	0.682	-1.204
Comt	0.723	1.102	0.971	-1.015	0.051	1.444
Cyb5r3	0.938	1.009	0.787	1.068	0.172	1.204
Cyp17a1	0.541	1.986	0.318	3.212	0.212	4.614
Cyp19a1	0.505	1.735	0.067	2.989	0.335	2.075
Cyp1a1	0.181	2.359	0.060	3.737	0.749	1.286
Cyp1a2	0.329	-4.691	0.323	-3.263	0.996	1.009
Cyp1b1	0.648	1.756	0.074	6.975	0.011	5.663
Cyp27b1	0.293	-3.666	0.541	-1.508	0.464	1.549
Cyp2b15	0.274	5.744	0.667	2.222	0.941	-1.123

Gene symbol	p value	Test /Control	p value	Test /Control	p value	Test /Control
	Rooibos		*C. genistoides*		*C. subternata*	
Cyp2b6	0.066	-5.651	0.131	-8.302	0.729	1.350
Cyp2c13	0.846	-1.197	0.092	6.678	0.062	3.886
Cyp2c6_predicted	0.548	-1.858	0.953	-1.075	0.179	5.547
Cyp2c7	0.553	1.218	0.189	1.581	0.099	1.641
Cyp2e1	0.507	-1.074	0.452	1.037	0.230	1.162
Cyp3a23/3a1	0.647	-2.027	0.764	-1.455	0.145	5.669
Cyp4b1	0.051	-2.193	0.070	1.882	0.183	1.519
Ephx1	0.088	-1.507	0.866	1.035	0.742	1.059
Faah	0.160	1.404	0.059	2.407	0.010	1.902
Fbp1	0.431	-1.144	0.871	1.032	0.689	1.072
Gad1	0.118	-9.511	0.338	-4.768	0.097	3.269
Gad2	0.857	1.202	0.251	3.111	0.655	1.383
Gckr	0.888	-1.190	0.286	2.462	0.092	3.727
Ggt1	0.024	-1.691	0.901	-1.031	0.169	-1.748
Gpi	0.258	1.250	0.846	-1.059	0.387	1.178
Gpx1	0.780	-1.051	0.415	1.195	0.182	1.327
Gpx2	0.791	-1.033	0.980	-1.007	0.790	1.040
Gpx3	0.462	1.167	0.998	-1.001	0.169	1.689
Gpx4	0.361	-1.073	0.748	-1.031	0.897	1.009
Gpx5	0.953	-1.121	0.772	-1.491	0.943	-1.102
Gsr	0.619	1.079	0.498	-1.142	0.753	-1.048
Gsta3	0.580	-1.090	0.437	-1.183	0.657	-1.063
Gsta4	0.320	-1.306	0.888	-1.053	0.498	-1.165
Gstm1	0.047	1.572	0.971	-1.008	0.049	1.693
Gstm2	0.223	1.225	0.712	1.105	0.150	1.337
Gstm3	0.278	1.092	0.037	-1.601	0.237	-1.273
Gstm4	0.629	-1.384	0.886	1.031	0.041	1.742
Gstm5	0.181	-1.226	0.021	-1.614	0.008	-1.691
Gstp1	0.329	1.194	0.361	-1.187	0.838	-1.067
Gstt1	0.412	-1.134	0.326	-1.163	0.200	-1.348
Hk2	0.121	-1.827	0.017	-2.119	0.013	-2.184
Hsd17b1	0.916	-1.028	0.661	1.079	0.356	-1.215
Hsd17b2	0.671	-1.195	0.825	1.105	0.577	1.260
Hsd17b3	0.267	134.314	0.057	4.424	0.760	1.359
Lpo_predicted	0.442	4.027	0.277	-7.487	0.179	11.307
Marcks	0.887	1.024	0.640	1.084	0.517	1.178
Mgst1	0.013	1.286	0.135	1.228	0.108	1.191
Mgst2_predicted	0.776	-1.094	0.347	1.658	0.039	2.615
Mgst3_predicted	0.128	-1.330	0.793	-1.070	0.905	1.017
Mpo	0.624	2.279	0.743	-1.539	0.055	18.598
Mt3	0.323	-2.258	0.350	-1.474	0.193	-2.023
Mthfr_predicted	0.380	-1.252	0.436	1.183	0.567	1.340
Nat1	0.015	-1.225	0.894	1.064	0.296	-1.074

Gene symbol	p value	Test /Control	p value	Test /Control	p value	Test /Control
	Rooibos		*C. genistoides*		*C. subternata*	
Nos2	0.731	1.450	0.791	1.730	0.651	1.426
Nos3	0.586	-1.119	0.272	1.286	0.196	1.500
Nqo1	0.215	1.122	0.071	1.680	0.219	1.248
Pklr	0.895	-1.018	0.272	-1.148	0.399	-1.114
Pkm2	0.088	1.217	0.652	1.097	0.232	1.116
Pon1	0.432	-3.979	0.196	-12.812	0.896	1.126
Pon2	0.773	-1.066	0.440	-1.086	0.157	1.185
Pon3	0.783	-1.028	0.324	1.260	0.485	-7.110
Smarcal1_predicted	0.140	-1.530	0.698	-1.171	0.509	1.155
Snn	0.065	-1.424	0.309	-1.327	0.089	-1.253
Srd5a1	0.782	1.034	0.108	1.386	0.095	1.215
Xdh	0.833	-1.091	0.713	1.167	0.396	1.489
Rplp1	0.844	-1.020	0.744	-1.032	0.863	1.013
Hprt	0.979	1.005	0.880	-1.028	0.687	-1.076
Rpl13a	0.139	-1.369	0.897	1.025	0.803	-1.058
Ldha	0.796	1.031	0.091	-1.217	0.746	-1.044
Actb	0.073	1.347	0.272	1.260	0.287	1.173

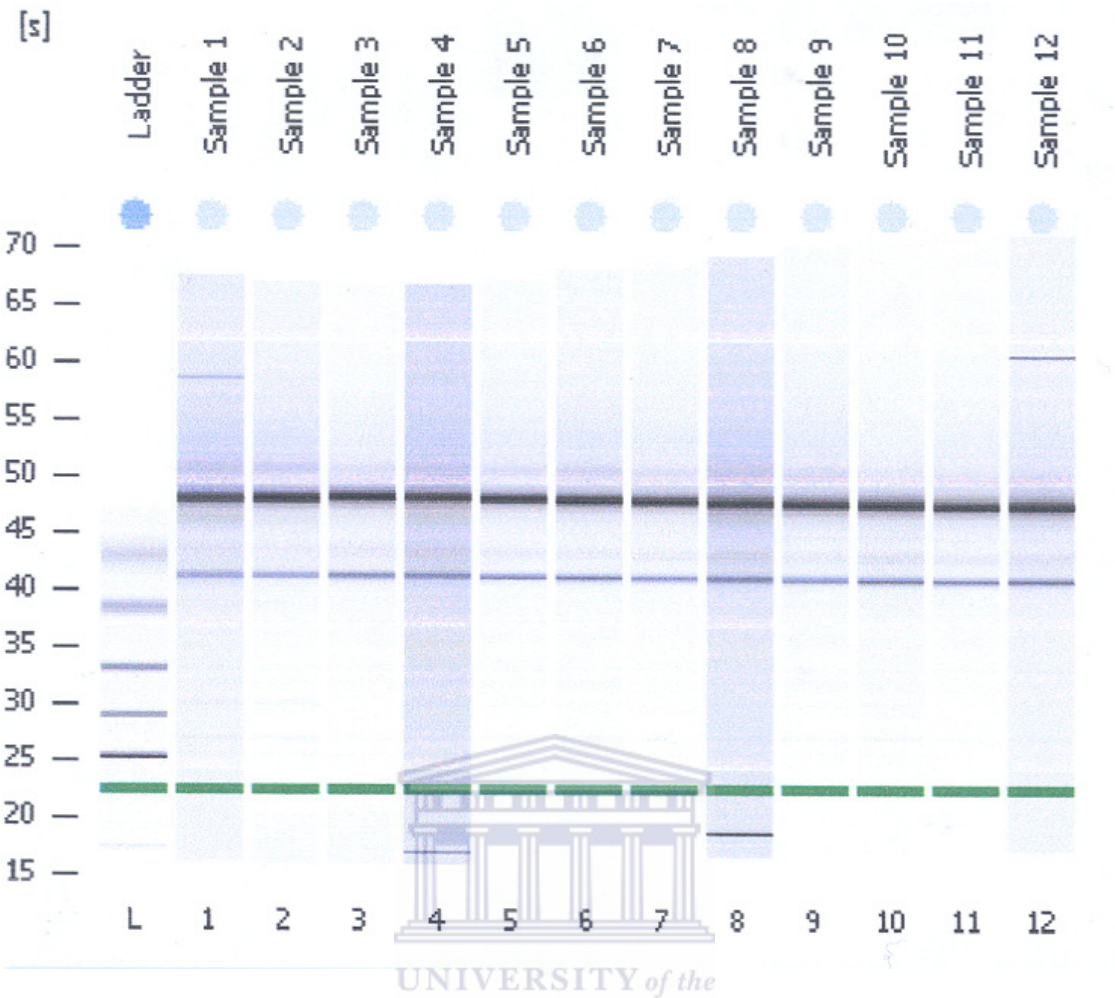

Figure 1 Distinct bands of the 28S and 18S ribosomal units.

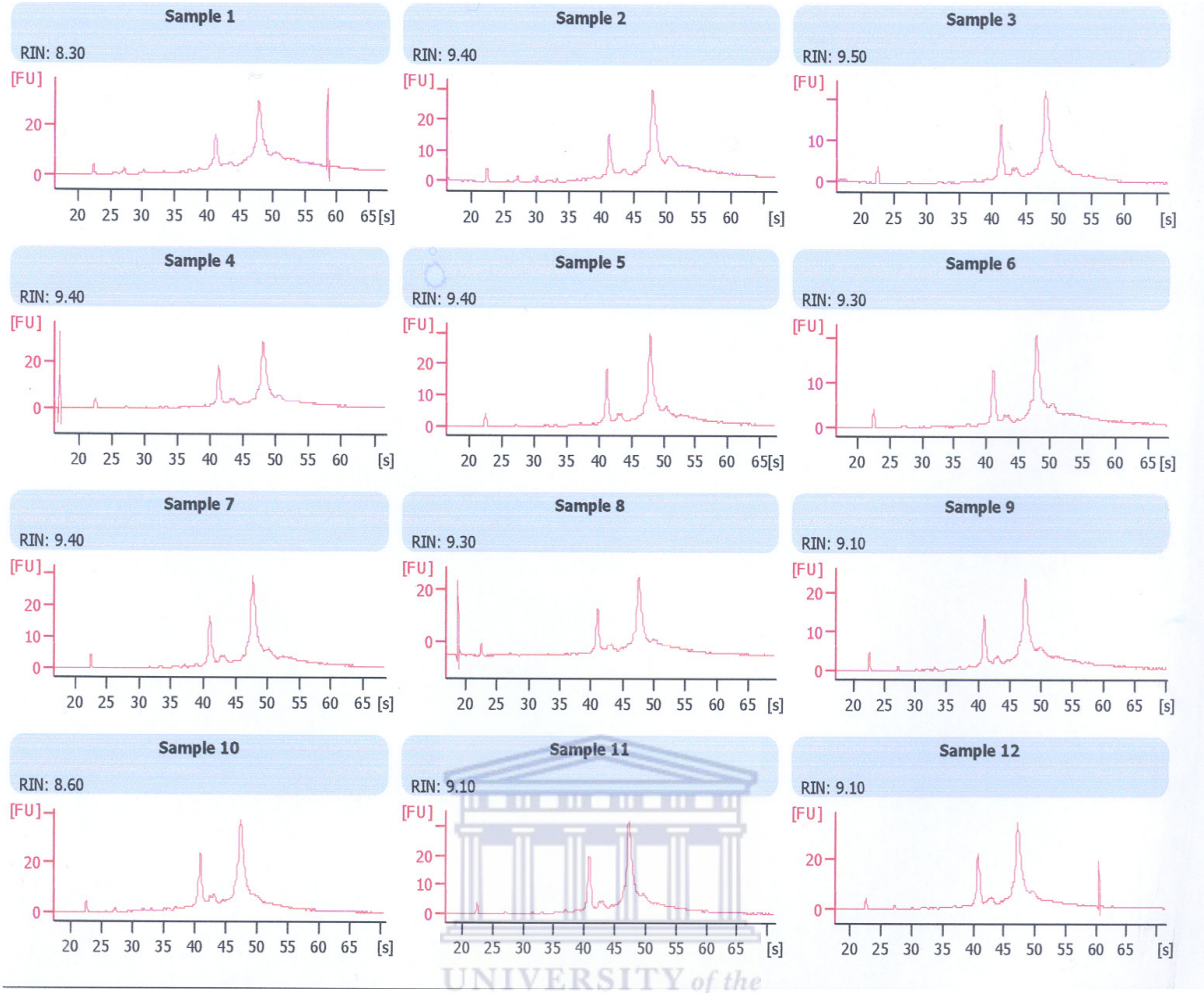

Figure 2 Distinct peaks of the 18S and 28S ribosomal units and RNA integrity numbers (RINs).

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Ethics Committee for Research on Animals (ECRA)

The Medical Research Council

PO Box 19001, Tygerberg, 7505, Cape Town, South Africa
off Hindle Road, Brentwood Park, Driftsands
Tel +27 (0)21 955 1900, Fax +27 (0)21 955 1330
E mail: gieliana.fourie@mrc.ac.za
Http://www.sahealthinfo.org/Modules/ethics/ethics.htm

17 April 2007

Ms Debora van der Merwe
Promec Unit
MEDICAL RESEARCH COUNCIL

Dear Ms van der Merwe,

YOUR REVISED APPLICATION TO THE ECRA : REF. 05/07 "Bioavailability of the major polyphenolic compounds in rooibos and honeybush".

The ECRA Committee received your revised application and it has been approved.

You may start with your experiment now.

May I remind you that you are required to submit a brief progress report on the progress of this study at six monthly intervals so the ECRA can be kept informed of the progress you are making and of any problems you may encounter.

Your progress report dates : November 2007 and May 2008.
We will remind you by letter.

Kind regards.

PROF D DU TOIT
Chairperson : ECRA Committee

ADDENDUM III

PREPARATION OF PRIMARY RAT HEPATOCYTES

Only use autoclaved ddH_20, autoclaved glassware and sterile equipment!

PERFUSION REAGENTS (STORED AT 4°C):

L-Gluthamine:
0.2 M (200 mM) in WE
1 M = 146.1 g/1000 mL
2 M = 292.2 g/1000 mL
0.2 M = 29.22 g/1000 mL
= 2.92 g/100 mL WE or 1.46 g/50 mL WE

L-Proline:
0.2 M (200 mM) in WE
1 M = 115.1 g/1000 mL
2 M = 230.2 g/1000 mL
0.2 M = 23.02 g/1000 mL
= 2.30 g/100 mL WE or 1.15 g/50 mL WE

Pyruvate (Na):
1 M in WE
1 M = 110.0 g/1000 mL
= 11.0 g/ 1000 mL
= 5.5 g/1000 mL

Insulin:
Dilute from liquid stock:
20 U/L in 6 mM HCl
25.6 U/mg
In 0.1 g (100 mg) = 2560 U…0.1g (100mg) contains 2560 U
2560 U in 25.6 mL [6 mM] HCl
≈ 100 U in 1.0 mL
Dilute stock with x 5 in WE when used in medium

EGTA:
0.05 M in HBSS
1.902 g in 100 mL HBSS + 3 NaOH pellets
pH = 7.4

Collagen:
3 mg/mL powder stock: 25 mg bottle + 8.333 mL HCL [0.012 M]
Place in water bath at 37°C to dissolve
For plating: dilute stock 1:4 with autoclaved ddH_2O

Or use liquid stock (dilute 1:4 with autoclaved ddH_2O)
Williams' Medium E:
1 bottle of WE
Add 2.2 g of $NaHCO_3$
Make up to **1 L** with autoclaved ddH_2O in a volumetric flask.
pH 7.2 (with HCl)
Add 5 mL Pen/Strep/Fungizone to this bottle (work sterile)

Hank's Balanced Salts:
1 bottle of HBSS
Set pH to 7.2 (with NaOH)
Add autoclaved ddH_2O up to 100 mL in a volumetric flask

Hank's wash:

Total volume	**250 mL**	**500 mL**
	25 mL HBSS	50 mL HBSS
	2.5 mL HEPES	5 mL HEPES
	222.5 mL ddH_2O	445 mL ddH_2O

Hank's perfusion:

Total volume	**250 mL**	**500 mL**
	25 mL HBSS	50 mL HBSS
	2.5 mL HEPES	5 mL HEPES
	2.5 mL EGTA	5 mL EGTA
	220 mL ddH_2O	440 ddH_2O

WE for perfusion:
Total volume ±25**0 mL**
250 mL WE
2.5 mL HEPES

WE + 10% FBS:

Total volume	**250 mL**	**500 mL**
	25 mL FBS	50 mL FBS
	2.5 mL HEPES	5 mL HEPES
	*2.5 mL Gluthamine	*5 mL Gluthamine
	**0.25 mL Insulin	**0.5 mL Insulin
	217.25 mL WE	434.5 mL WE

WEX with 0.5% FBS:

Total volume:	**250 mL**	**500 mL**
	2.5 mL HEPES	5 mL HEPES
	1.25 mL FBS	2.5 mL FBS
	*2.5 mL Gluthamine	*5 mL Gluthamine
	2.5 mL Pyruvate	5 mL Pyruvate
	2.5 mL Proline	5 mL Proline

**0.25 mL Insulin	**0.5 mL Insulin
237.25 mL WE	474.5 mL WE

Filter reagents in the following order: "Hanks' Wash", "Hanks' Perfusion", "WE for perfusion", "WEX" and "WE + 10% FBS". Use a 1 L Corning filter system.

PRIMARY RAT HEPATOCYTE PERFUSION TECHNIQUE

- Autoclave a glass petri dish or take a sterile plastic petri dish to Room 330, this is used to transport the perfused live to the Culture Room
- Coat the petri dishes
- Make sure there are sterile cheese cloth filters (single layer and double layer). Make sure there a plastic bags and hand towels available for the perfusion
- Make sure there are a spray bottle with 70% ETOH available

Perfusion pump and tubing:

- ALWAYS leave the tubing in 70% ETOH
- Make sure there are autoclaved ddH_2O water to rinse the tubing
- Once you have changed the setting of the tubing on the pump you need to calibrate the pump flow to 15mL/min
- Check pump flow before you do a perfusion use a measuring cylinder

Filter reagents in the following order: "Hanks' Wash", "Hanks' Perfusion", "WE for perfusion", "WEX" and "WE + 10% FBS". Use a 1 L Corning filter system.

- 125 mg Collagenase (weigh out separately in glass bijous or blue 15 mL falcon tube): Keep on ice:
- 118 mg $CaC_{L2}\cdot 2_{H2}O$ (weigh out separately in glass bijous or blue 15 mL falcon tube): Keep on ice:___
- Rinse perfusion pump well with 70% ETOH
- Rinse perfusion pump with ddH_2O
- Switch on all the water baths (37°C)
- Sterilise all working areas
- Place All the reagents in a water bath (at 37°C)
- Wash the petri dishes x 1 with Hank's wash
- Sterilise all the instruments
- After perfusion pump is thoroughly cleaned: Start pumping Hank's perfusion solution.

Use rats between 150 and 300 g
Anaesthetise rats: (0.22 pentobarbital /100g body weight).

1. Check temperature of all water baths continuously during the perfusion
2. Reduce pump speed (0.15)
3. Spray rat with 70% ETOH
4. Cut 2X catgut lengths (± 10 cm)
5. Remove skin from a big area
6. Make sure hair will not come into contact with the insides of the rat
7. Rinse all instruments to remove hair
8. Cut open muscle up to the diaphragm – carefully, do not puncture diaphragm
9. Move intestines with gauss swaps, wipe to the right – be gentle
10. Expose portal and aorta veins
11. Do not touch liver with hands use the swaps
12. Carefully pull catgut around each vein and bind loosely
13. Insert perfusion needle into portal vein
14. Tie catgut around the needlepoint – rather tightly
15. Increase pump flow to a set mark to ensure a flow of 15 mL/min
16. Tie off aorta catgut string
17. Open the thorax and puncture the heart
18. Liver should loose color immediately
19. Discoloration (blood removal) should be visible over all the liver lobes
20. Place muscle top layer over the perfused liver
21. Perfuse for 10 min
22. After 5 min add the Collegenase and $CaCL_2$ to the WE for perfusion
23. Mix well until all the Collagnenase has dissolved
24. Stop the pump and put pump tube into the WE for perfusion
25. Start the pump and wait until a darker pink are observed in tubes before starting the timer
26. Digest for ± 13 min at speed between 5.00 and 4.00
27. Remove top muscle layer and expose liver
28. Carefully and lightly stroke liver without using your hands
29. If a bubble is observed try to perfuse as long as possible don't wait until it bursts
30. Check liver closely, stop perfusion if liver cell separation under the capsule is visible
31. After digestion stop pump and remove needle
32. Hold liver over a big petri dish and cut loose
33. Pour some WE for perfusion onto the liver
34. Proceed to the sterile laminar cabinet immediately
35. Add WE + FBS 10% immediately to inactivate the enzyme

Wash pump with autoclaved ddH2O followed by 70 % ETOH, leave it in the 70 % ETOH

Cell harvesting and growth:

1. Remove liver capsule with tweezers
2. Gently free the cells from the capsule
3. Filter through a sterile single layer cheesecloth into a 50 mL blue cap Falcon tube.
4. Rinse with **WE + 10% FBS.**
5. Centrifuge for 10 min at 450 rpm.
6. Remove top supernatant.
7. Fill tube with **WE + 10% FBS:** 20 mL for a 5 mL pellet.
8. Gently "dissolve" cells in this solution.
9. ALTERNITIVE METHOD: use a shaking water bath set at 37°C to dissolve cells (± 20 min) in the blue–top falcon tube. Wrap the cap in parafilm to ensure no water leaks in.
10. Filter through the double cheesecloth layer into another 50 mL tube – Do not rinse!
11. This solution is used for the viability determinations.

Viability determinations:

0.5 mL cell (gently mix cells suspended in the WE + 10% FBS solution to homogenate)
4.5 mL WE
1 mL Trypan blue
(Ensuring you have a 12X dilution)

Wait 5 min.

Place in heamocytometer.
Count cells in each of the 4 squares and calculate the mean.
Count live cells: colorless with a distinct outline.
⇒Single cells or strings indicate the perfusion was a success.
⇒Clumps of cells indicate a bad perfusion.
Count the dead cells**:** cells will be blue in color with a blue nucleus.

% Viability: dead cells/ Total cell count (living +dead cells) x 100
= (100 – answer)
= % viable
Value above 90% is very good and preferable
Plate according to the no living/viable cells

Determining the dilution needed for plating the cells:

Example:

Use 60 mm petri-dishes
Need 40 dishes
6 mL "WE + 10% FBS" needed per 60 mm petri-dish
Plate 6 x 10^5 cells/ 6 mL "WE + 10% FBS" (one petri-dish)
After counting viable cells work out the cells in suspension (cells/mL) =
Mean (of 4 squares) = 35

- cells/mL = 35 cells / mm^2
  = 350 / mm^3 (depth of heamocytometer = 0.1 mm)
  = 35 x 10^4 x 12
  = 420 x 10^4
  = 4.2 x 10^6

For 40 dishes:

240 x 10^5 cell needed
= 24 x 10^6 cells
$\therefore$ 4.2 cells/mL = 24 x 10^6 cells/X
X = 24 x 10^6/4.2 x 10^6
X = 5.7 mL

Aliquot 5.7 mL of cell suspension and add to 240 mL "WE + 10% FBS" (40 x 6mL media)

Coating of Petri dishes:

Collagen should be room temperature.
Place in water bath at 37°C!
Dilute the collagen 1:4 = 1 part collagen and 4 parts autoclaved ddH_2O
2 mL diluted collagen solution can be used to coat 20 small (35 mm) petri dishes.
Place 1 mL (2 mL) diluted solution in small (60 mm) dishes and leave for 1 min
Remove and place in next dish.
Place in Incubator overnight
Wash x 1 next morning with Hank's wash: 1 mL for small and 2 mL for big.

96 well microtitre plates:

Use same diluted collagen solution.
Only use 100 µL for each well. Also transport from one (or one row with the aid of a multi-pipette) to the next. Wait 30-60 sec.

Plating of cells:

Place the petri dishes in a incubator for a minimum of 2 hours.
Remove the WE + 10% FBS by washing the plates 2x with Hank's wash.

(FBS may mask any toxic effect of other reagents added – not good when investigating cytotoxicity of compounds)
Add 2mL or 3mL WEX receptively to the 35 mm and 60 mm plates.

While plating and washing, continuously monitor the cell adhesion to the collagen. No use in doing the experiment if you lost all the cells while removing media or washing.

Rat liver perfusion stock reagents

Reagent	Stock Storage	Working Stock Storage	Specifications	Company
WE	2-8 ℃ Fridge Culture room	N/A	- L-Glutamine; - $H(CO_3)_2$	Sigma: W4125 10 x 1 L
WE plus GlutaMAX	2-8 ℃ Fridge Culture room	N/A	+ L-Glutamine; +$H(CO_3)_2$	Invitrogen/Gibco 32551 – 020 for 500 mL 32551-087 10x500 mL
HBSS	2-8 ℃ Fridge Culture room	N/A	- Ca, Mg, Phenol red, $Na(CO_3)_2$ 500 mL	Sigma: H2387 10 x 1L
HEPES	2-8 ℃ Fridge Culture room	2-8 ℃ Fridge Culture room	1M 100 mL	Invitrogen/Gibco 15630
FBS	-20 ℃ Culture room	N/A	Heat inactivation required Aliquot in 50 mL	INV/GIBCO 10106
Collagen	Powder: -20 ℃ Liquid: Fridge Culture room	After Diluted: 2-8 ℃ Fridge Culture room	Type 1 from rat tail	Powder: Sigma: C7661 Liquid: Sigma: C3867-1VL
Collagenase	-20℃ Culture room	N/A	Type IV	Sigma: C5138
Pen/Strep/Fungizone (Amphotericin B)	-20 ℃ Culture room	N/A	Aliquot in 5 mL	BioWhittaker/Lonza 17-745E
Insulin	-20 ℃ Culture room	2-8 ℃ Fridge Culture room	From Bovine Pancreas 100mg	Sigma: I1882
Sodium-pyruvic acid	Powder: Room temp Lab 312 Liquid: -20 ℃ Culture room	2-8 ℃ Fridge Culture room	Powder: 1 M Liquid: 100 mM (100 mL)	Powder: Sigma: P5280 Liquid: BioWhittaker/Lonza: 13-115E
L-Glutamine	Powder: Room 312 Liquid: -20 ℃, Culture room	2-8 ℃ Fridge Culture room	200 mM 100 mL	Powder: Sigma: G8540 Liquid: BioWhittaker/Lonza 17-605E
L-Proline	Room 312	2-8 ℃ Fridge Culture room		Powder Sigma: P5607

MEM Non-essential Amino Acid Solution	Culture Room	-20℃ Freezer Culture Room	Used instead of L-proline	BioWhittaker/Lonza 13-114E
EGTA	Room 312	2-8 ℃ Fridge Culture room		Sigma: E3889
$NaHCO_3$	Room 312	N/A		Sigma: S5761
$CaCl_2 2H_2O$	Room 312	N/A		ICN: 193818
Corning filter system	Culture Room	N/A	1 L 0.22 µm PES	Sigma: CLS431098
Perfusion needle Canula with injection valve PTVE	Room 312	Room 229	G: 18 Ø: 1.3 mm L 45 mm Flow: 90 mL/min	Rob Dyer: Neotec Medical Industries Ref: NM-218
Surgical Catgut Chromic	Room 229		Sterile 3.5 m 150 cm each	Rob Dyer: SCIMITAR Surgical Sutures Ref CG 290 Ligature/ CG20-150

CONFERENCES AND SEMINARS

2008 Presented a poster at the Pan-African Environmental Mutagen Society (PAEMS), an international conference held at the CTICC, Cape Town.

2009 Presented and was awarded the best poster award at the Indigenous Plant Use Forum (IPUF), held in Stellenbosch.

2009 Attended a training seminar on laboratory safety and chemical grades at the University of the Western Cape.

2010 Presented and was awarded third best oral presentation at the IPUF conference, held in Keimoes, Upington.

2010 Presented at the MRC research day, held in Parow, Cape Town.