The inhibitory effect of Rooibos on cytochromes P450 and downstream *in vitro* modulation of steroid hormones

by

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Declaration

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Abstract

This study describes:

1. Substrate binding assays investigating the effects of methanolic extracts of unfermented and fermented Rooibos on the binding of natural substrates to ovine adrenal microsomal and mitochondrial P450 enzymes, demonstrating the interference of substrate binding in the presence of the Rooibos extracts.

2. The effects of selected flavonoids (quercetin, rutin and aspalathin) on the binding of natural substrates to ovine adrenal microsomal and mitochondrial P450 enzymes, demonstrating interference of substrate binding in the presence of the flavonoid compounds.

3. Substrate conversion assays in non-steroidogenic COS-1 cells to investigate the effects of methanolic extracts of unfermented and fermented Rooibos on the activity of key steroidogenic P450 enzymes (CYP17A1, CYP21A2, CYP11B1, and CYP11B2), demonstrating inhibition of the catalytic activity in the presence of Rooibos extracts.

4. The effects of selected flavonoids on the substrate conversion of the aforementioned key steroidogenic enzymes expressed in COS-1 cells.

5. An investigation of the effect of methanolic extracts of unfermented and fermented Rooibos on steroid hormone production in human adrenal H295R cells under basal and stimulated conditions, demonstrating the modulating effects of unfermented and fermented Rooibos extracts. Basal and stimulated steroid hormone production was decreased in the presence of unfermented and fermented Rooibos.

Opsomming

Hierdie studie beskryf:

1. Die gebruik van substraatbindings-essais om die effek van metanoliese ekstrakte, van gefermenteerde- en ongefermenteerde Rooibos, op die binding van die natuurlike substrate aan skaap adrenale mikrosomale en -mitochondriale P450 ensieme te bepaal. Daar is getoon dat die ekstrakte 'n beduidende inhiberende effek op ensiem-substraatinteraksie gehad het.

2. Die die inhiberende effek van geselekteerde flavonoïede (kwersetien, rutien and aspalatien) op die binding van die natuurlike substrate aan skaap adrenale mikrosomale en -mitochondriale P450 ensieme.

3. Die gebruik van substraatomsettings-essais in nie-steroïedogeniese COS-1 selle, om die effek van gefermenteerde- en ongefermenteerde Rooibos ekstrakte op die aktiwiteit van die steroïedogeniese P450 ensieme (CYP17A1, CYP21A2, CYP11B1, and CYP11B2) se katalitiese aktiwiteit te bepaal. Daar kon aangetoon word dat die katalitise aktiwiteite van bg. ensieme beduidend beïnvloed word deur die Rooibos ekstrakte.

4. Die gebruik van substraatomsettings-essais in nie-steroïedogeniese COS-1 selle, om die effek van geselekteerde flavonoïede op die aktiwiteit van bogenoemde steroïedogeniese P450 ensieme te bepaal.

5. 'n Ondersoek na die invloed van metanoliese ekstrakte van gefermenteerde- en ongefermenteerde Rooibos op steroïedhormoon biosintese in die menslike adrenale H295R-selmodel. Die ondersoek, onder basale en gestimuleerde toestande, het getoon dat beide Rooibosekstrakte in bogenoemde toestande steroïedhormoon produksie geinhibeer het.

Dedication

First and foremost l would like to dedicate this work to God the Almighty, l could not have done it without Him. My mother, Virginia Mugari, for her encouragement, unconditional love, her faith in me and contribution in the writing of this manuscript. My brothers, Tinotenda and Jimmy Mugari, for their support. My aunt and uncle, Dr Joyce Mujuru and Mr Evans Maphenduka, for being father figures and for always believing in me.

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Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase
11βHSD	11β-hydroxysteroid dehydrogenase
11DHC	11-dehydrocorticosterone
11βOH-A4	11β-hydroxyandrostenedione
16-OHPROG	16-hydroxyprogesterone
17-OHPREG	17-hydroxypregnenolone
17-OHPROG	17-hydroxyprogesterone
17βHSD	17β-hydroxysteroid dehydrogenase
A4	androstenedione
ACAT	cholesterol acyltransferase
ACE	angiotensin-converting enzyme
ACTH	adrenocorticotropic hormone
ADX/ Fdx	adrenodoxin
ADXR/ FdR	adrenodoxin reductase
AMP	adenosine monophosphate
ANOVA	analysis of variance
ALDO	aldosterone
Ang II	angiotensin II
AT_1	angiotensin II receptor type 1

ATP	adenosine triphosphate
AVP	arginine vasopressin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CE	cholesterol esters
CEH	cholesterol esters hydroxylase
CoA	coenzyme A
COS-1	transformed African green monkey kidney tumour cells
CORT	corticosterone
CRH	corticotrophin releasing hormone
CYP101	cytochrome P450cam
CYP11A1	cytochrome P450 side-chain cleavage
CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17A1	cytochrome P450 17α-hydroxylase/17,20 lyase
CYP21A2	cytochrome P450 21-hydroxylase
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DMEM	dulbecco's modified eagle's medium
DMEM/F12	dulbecco's modified eagle medium: nutrient mixture F-12
DOC	deoxycorticosterone

DPPH	1-diphenyl-2-picrylhydrazyl radical
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide or adrenodoxin reductase
FC	free cholesterol
FMN	flavin mononucleotide
FSH	follicle-stimulating hormone
H295R	human adrenocarcionoma cell line
HDL	high density-lipoproteins
HPA axis	hypothalamic-pituitary-adrenal axis
HPLC-DAD	high-performance liquid chromatography with diode-array detection
HRE	hormone respone element
HSDs	hydroxysteroid dehydrogenases
HSP	heat shock protein
LBD l	ligand-binding domain
LDL	low-density lipoproteins
LH	luteinizing hormone
UPLC-MS/MS	ultra-performance liquid chromatography tandem mass-spectrometry
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate

PBR	peripheral type benzodiazepine receptor
PEG	polyethylene glycol
PKA	protein kinase
POR	cytochrome P450 oxidoreductase
PREG	pregnenolone
PROG	progesterone
RAAS	renin-angiotensin-aldosterone system
SEM	standard error of the mean
SRs	steroid receptors
StAR	steroidogenic acute regulatory protein
T-cells	type of lymphocyte white blood cell
TPA	12-O-tetra-decanoylphorbol-13-acetate
TSH	thyroid-stimulating hormone

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CHAPTER 1

Introduction and objectives

1.1. Introduction

The traditional use of medicinal plants for therapeutic purposes in the maintenance of good health and in the treatment of diseases has been on-going for centuries. Southern Africa is an important focal point of botanical and cultural diversity but only a few species of plants have been commercialized as herbal or medicinal products (Van Wyk 2011). One of the Southern African plants that have gained local and global popularity is *Aspalathus linearis* generally known as Rooibos.

Rooibos is unique to the Western Cape province of South Africa. It is commonly consumed as a beverage. It is processed in two different forms, fermented and unfermented Rooibos, both of which are commercially available. Rooibos is currently processed and sold in various forms, such as lotions, skin care products, ice tea and, most recently, as a red cappuccino. Although Rooibos is enjoyed as a "tea" competing in the local market with coffee and *Camellia Sinensis*, it is popular as a herbal beverage in the international markets due to its health giving properties and high anti-oxidant content. Rooibos contains active compounds such as polyphenols to which the anti-oxidant activity is ascribed. Anti-oxidant activity and flavonoid content differ in the two forms of Rooibos with unfermented Rooibos having higher levels of flavonoids and greater anti-oxidant activity than fermented Rooibos. These differences are attributed to the fermentation process to which Rooibos is subjected after harvesting in order to acquire its unique sweet fruity flavour and colour (Joubert et al. 2011).

Anecdotally, Rooibos has been reported to be used in treating ailments related to the endocrine system such as anxiety, stress, hypertension, and diabetes. Many of these ailments result from imbalances in the endocrine system abnormal adrenal hormone levels. The adrenal glands are situated on top of each kidney and are crucial for physiological homeostasis. Hormonal imbalances are associated with various clinical conditions which include, amongst others, cardiovascular diseases, diabetes, hypertension and metabolic syndrome. Steroid hormone biosynthesis is catalysed by cytochrome P450 enzymes which are heme monoxygenases expressed in the adrenal gland as well as the steroid

dehydrogenases which include 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase (3βHSD and 17βHSD). These enzymes catalyse the biosynthesis of mineralocorticoids, glucocorticoids, and androgens (see Chapter 3).

1.2. Methodology

First, the cytochrome P450 11β -hydroxylase (CYP11B1), aldosterone synthase (CYP11B2), cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17A1) and cytochrome P450 21-hydroxylase (CYP21A2) and their role in steroid biosynthesis in the adrenal gland were discussed (Chapter 3). The unique spectral properties of these enzymes were used to investigate the effect of Rooibos and flavonoid compounds present in Rooibos on the binding of steroid substrates to the adrenal P450 enzymes (Chapter 4). Spectral binding assays were performed using ovine microsomal and mitochondrial preparations with progesterone (PROG) and deoxycorticosterone (DOC) as substrates. PROG is the substrate for both CYP17A1 and CYP21A2 present in the microsomal preparations while in the mitochondrial preparations, DOC is substrate for CYP11B1 and CYP11B2. The microsomal and mitochondrial P450 enzymes do not only differ with respect to their cell organelle location, but also have different electron transport systems (as is discussed in Chapter 3).

The influence of Rooibos and the selected flavonoids on the catalytic activity of the P450 enzymes which catalyse adrenal steroid hormone biosynthesis was investigated. Substrate conversion assays were carried out in COS-1 cells, a non-steroidogenic mammalian cell line, in which the P450 enzymes were heterologously expressed. The influence of Rooibos extracts were subsequently investigated in H295R cells, an adrenal cell model, to determine the effect on the overall steroidogenic output (Chapter 5).

1.3. Aims of the study

The aims of the study are summarised below:

- To investigate the influence of unfermented and fermented Rooibos extracts and selected flavonoids on substrate binding to P450 enzymes in microsomal and mitochondrial preparations.

- To investigate the influence of unfermented and fermented Rooibos extracts and selected flavonoids on the catalytic activity of P450 enzymes expressed in COS-1 cells.
- To investigate the influence of unfermented and fermented Rooibos extracts on steroidogenesis in a human adrenal cell model (H295R).

1.4. Layout of the document

Chapter 1 comprises a brief introduction of the study, the aims of the study, and the methodology followed.

A detailed overview of Rooibos and flavonoids is presented in Chapter 2. Chapter 2 also presents reported clinical effects of Rooibos on *in vitro* and *in vivo* investigations.

An overview of P450 enzymes that catalyse steroid hormone biosynthesis and their role in steroid biosynthesis in the adrenal glands is presented in Chapter 3.

Chapter 4 addresses the use of the unique spectral properties of P450 enzymes and the use thereof to investigate the effect of Rooibos and selected flavonoid compounds present in Rooibos on the binding of steroid substrates to the adrenal P450 enzymes.

Chapter 5 describes an investigation into the influence of Rooibos and the selected flavonoids on the catalytic activity of the P450 enzymes that catalyse adrenal steroid hormone biosynthesis. The influence of Rooibos extracts on H295R cells, an adrenal cell model, was also investigated and described in Chapter 5, to determine the effect on the overall steroidogenic output.

Chapter 6 comprises of the summary and conclusions of results obtained in the study. Future work investigations for the continuation of the study are suggested in Chapter 6.

CHAPTER 2

Rooibos (*Aspalathus linearis)*.

2.1. Introduction

Plants have been used for medicinal purposes for centuries; most traditional cultures depend on plants not only for food but for medicine. Herbal preparations have been typically prepared by heating the plant material in water to produce an extract that could be used as a herbal beverage (tisane) or ointment. Plants are considered to induce fewer side effects and less toxic than synthetic drugs. According to the World Health Organisation, 80 % of the world's population use plant extracts, often in aqueous solutions, for their primary health care. Herbal preparations have been used for centuries as tisanes in Europe and Asia (Loew & Kaszkin 2002; Patel et al. 2012). Such plants include the *Camella sinensis, Cyclopia* (honey bush tea) and Rooibos (Figure 2.1). These plants have been used for medicinal purposes based on anecdotal evidence rather than scientific evidence.

Figure 2.1. Rooibos plantation (reproduced from www.newlands.ca).

Rooibos's botanical name is *Aspalathus linearis* (*A. linearis*), family – Fabaceae (pea family), tribe Crotalarieae (woody shrubs). The plant species is endemic to the Cape Flora. Rooibos is a leguminous plant indigenous to South Africa, is now popular on the international market as a caffeine-free beverage with low alkaloid tannins and seemingly poises as an elixir of life. Rooibos production is one of the most successful and important indigenous industries in South Africa (Van Wyk 2011). Rooibos is cultivated and produced

in the Western Cape. Nowadays it is not only consumed in South Africa but also exported to the rest of the world because of its popularity and international demand (Joubert et al. 2008). The use of *A. linearis* as a tisane by the Khoisan was first reported by Carl Thunberg in 1772 (Hawkins et al. 1983). The Khoisan crudely processed Rooibos plant material in warm summer months by bruising it and leaving it to ferment in heaps and then drying it in the sun. In 1904, Benjamin Ginsberg, a merchant of Clanwilliam and the first to exploit Rooibos commercially started trading with it in Cederberg. Carl Thunberg noted that the Khoisan people used Rooibos as a medicinal plant and referred to Rooibos as Rooibosch or Rooitee. Its medicinal value was only realised by P. Le Fras Nortier, a medical practitioner and nature lover from Clanwilliam in 1930 (Joubert & de Beer 2011). It was marketed in 1904, in its fermented form, that is, the oxidised form and, only recently Rooibos is now on the market in its unfermented form which is the un-oxidised form of Rooibos (McKay & Blumberg 2007; Joubert et al. 2008).

Rooibos is an erect or prostrate plant, which grows up to 152.4 cm (Figure 2.1). The plant is easily recognisable by its woody red stem, needle-like leaves and small yellow pea-like flowers as shown in Figure 2.2 (van der Merwe et al. 2006; Erickson 2002). Rooibos is adapted to poor nutrient, sandy, and acidic soils as it has roots that extend more than 200 cm deep into the soil to reach soil water. It has root nodules that contain bacteria which coverts nitrogen into nitrates to nourish the plant and the plant, in turn, provides carbohydrates and shelter for the bacteria (Hawkins et al. 1983; Kanu et al. 2013). It regenerates by re-sprouting or re-seeding after fire and it plays an ecological role in nitrogen-fixing (van der Bank, 1999; Cocks & Stock, 2001). Cultivation of the plant is susceptible to attacks from fungal disease (Neocosmospora vasinfecta and Diaporthe phaseolorum) and insect attacks such as the clearwing moth. Control of the attacks by chemical spraying is not desirable as the demand for organically produced Rooibos is high; thus developments of integrated pest management which include biological control management is currently being developed (van der Merwe et al. 2006). The plant's leaves and stems go through two manufacturing processes to produce unfermented and fermented Rooibos. The traditional and modern processing fermentation methods gives the fermented Rooibos its unique red brown color and sweet taste, while the unfermented Rooibos or "green" Rooibos is obtained by preserving the green leaves and stems, and ensuring oxidative changes are kept to a minimum (Joubert et al. 2008).

Figure 2.2. Rooibos plant (*A.linearis*) (reproduced from of www.anniesremedy.com)

As shown in Figure 2.3, Rooibos seedlings are grown in a nursery from hand collected seeds from healthy mature Rooibos plants in late January. The seedlings are transplanted into fields by hand or planting machines from the nursery when they have reached a specific height (10-20 cm). Rooibos is harvested during summer months and early autumn, preferably no flowers should be present as they give an uncharacteristic flavor to the Rooibos tisane. Harvesting is done manually using sickles or mechanically by cutting only the top half of the plant when it has grown 150 cm in height (Van Niekerk & Viljoen 2008).

After harvesting the shredded branches are bound and left overnight until it is processed the following day to wither the leaves. Processing of the tea involves batch processing which enables product flavor differentiation for a specific market (Joubert & Müller 1997). Processing involves either bruising (during which water is added); fermenting and drying in the sun after harvesting to produce fermented Rooibos, or immediately drying the Rooibos in the sun after harvesting to produce unfermented Rooibos (Figure 2.3). Bruising whilst adding water is necessary to accelerate fermentation initiated during shredding, also the water extracts polyphenols that will give colour the stems when absorbed. Pre-drying some plant material in the sun after harvesting inactivates enzymes responsible for the fermentation step thus skipping the fermentation step. The skipping of the fermentation step retains the green colour and flavonoid content of the Rooibos, particularly asphalathin (Joubert et al. 2011). For fermentation to take place, the shredded and bruised Rooibos is placed into heaps in 38 - 42 ° C ovens where the fermented Rooibos develops its unique red colour and sweet taste [www.klipopmekaar.co.za]. The plant material is spread out in the sun to dry after

fermentation, sieved to obtain the required fine cut, steam pasteurised and packaged. The unfermented Rooibos also undergoes steam pasteurisation before it is packed. Steam pasteurisation before packing was introduced after Salmonella contamination was reported (Joubert et al. 2011).

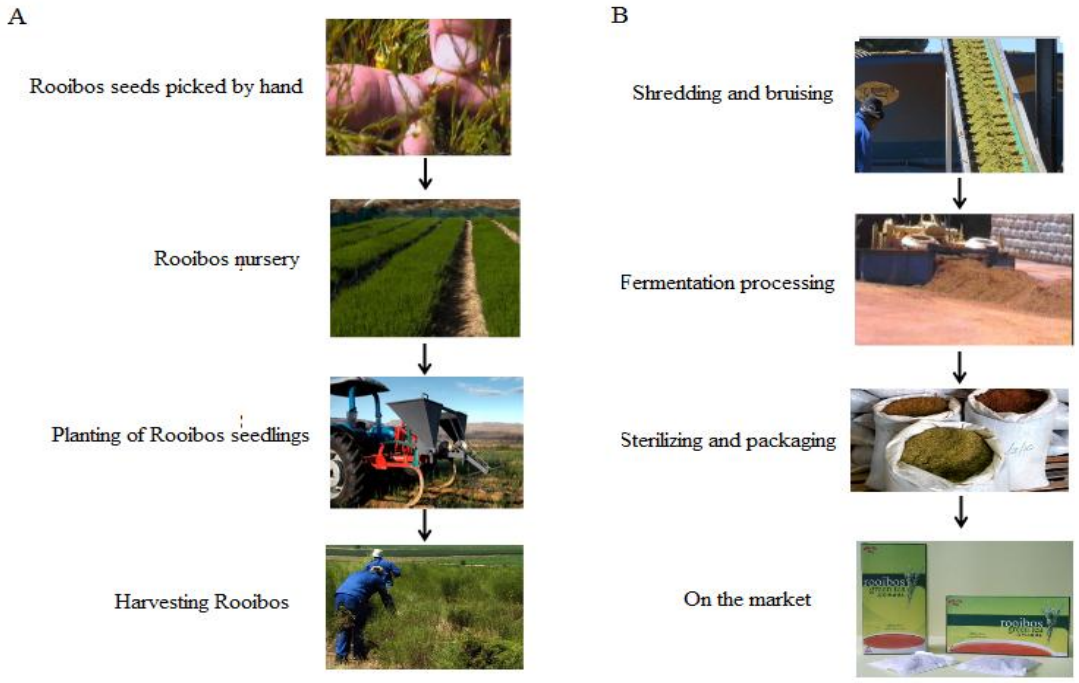


Figure 2.3. Rooibos plantation A) planting and harvesting process B) processing and packaging stages.

Rooibos contains secondary plant metabolites that exhibit biological activities contributing to its health giving properties. The beneficial properties of Rooibos are linked to the unique phenolic constituents in the plant, some of which are modified enzymatically during fermentation (Heerden et al. 2006). Flavonoids form the largest class of polyphenols and are natural metabolites that are ubiquitous in plants. Flavonoids are classified into groups according to their carbon skeleton properties. Flavonoids have the diphenylpropane carbon skeleton and have potent biological activities in the prevention of chronic illnesses (Rice-Evans et al. 1996; Scalbert & Williamson 2000; Gross 2004). Due to the processing methods, the flavonoid composition of the unfermented Rooibos differs from that of the fermented Rooibos (Standley et al. 2001). Flavonoids occur in *A. linearis* and the predominant polyphenols are the dihydrochalcones, flavanols and flavones (Joubert & de Beer 2011). Flavonoids have been found to have anti-inflammatory, anti-oxidant, anti-mutagenic, anti-

tumor, anti-fungal and anti-viral activities. The flavonoids have been reported to be responsible for the anti-oxidant activity of Rooibos (Arct & Pytkowska 2008).

Unfermented Rooibos has been reported to exhibit a higher anti-oxidant activity than the fermented Rooibos, attributed to the phenolic composition. Some flavonoids also have phytoestrogenic activity due to their structural similarities with steroid hormones (Mesiano et al. 1999). Green tea and black tea have been extensively studied for their health giving properties and, in particular the chemopreventive properties (van der Merwe et al. 2006). Some Rooibos studies that have been done, show that Rooibos has health promoting properties as it alleviates stress, anxiety, nervous tension and insomnia (Van Niekerk & Viljoen 2008). Most importantly, Rooibos has been reported to be safe for consumption by infants and pregnant women and, no side effects have linked to the consumption of Rooibos have been reported (McKay & Blumberg 2007). There are still more studies yet to be done to give more information on the Rooibos's health properties.

2.2. Flavonoids and their chemical structure and classes.

Flavonoids are a diverse group of secondary metabolites which are common in plant based foods such as wine, soya, vegetables, fruits, cereals and, herbal infusions (Hanhineva et al. 2010). They are plant chemicals (phytochemicals) responsible for plant pigmentation. Concentrations of flavonoids vary from plant to plant, plant's environmental factors and how the plant will be processed to make different types of flavours and colours (Moline et al. 2000). It is understood that flavonoids are not nutrients as they are not essential for life and usually occur in small amounts in plants. Physiologically, flavonoids act as anti-oxidants and screen for harmful radiation in plant tissues (Hashimoto & Tajima 1980; Rice-Evans et al. 1996). They also protect the plant from insect diseases, infestations and oxidative injury (Harborne & William 2000). Humans and animals do not have the ability to synthesise flavonoids making it important to have flavonoids in their diets (Yao et al. 2004). They are important for biological activities as they influence physiological or cellular activities that result in a beneficial health effect in mammals, thus they are also referred to as bioactive compounds.

Flavonoids contain at least one benzene ring and a pyran ring with one or more hydroxyl groups together with other substituents (Xiao & Kai 2012). The two aromatic rings are

connected together by a three carbon bridge (Figure 2.4A). Flavonoids are classified according to the numbering of the carbon skeleton as previously mentioned in the introduction. A basic flavonoid skeleton can have a number of substituents such as hydroxyl, isopentyl and methyl groups. Water solubility and lipophilic nature of flavonoids is determined by the hydroxyl groups and methyl groups respectively (Crozier et al. 2009). In addition to the numbering of the carbon skeleton, the substitutions and functional groups on different positions of the skeleton enable flavonoids to be put into distinctive groups as shown in section 2.3, Table 2.1. Flavonoids and their metabolites' biological activities depend on their chemical orientation and structure of their moieties (Yao et al. 2004). Some of the flavonoids exist as glycosides; molecules in which a sugar is attached to a non-carbohydrate moiety (small organic molecule). Most plants store chemicals as inactive glycosides which are then activated by enzyme hydrolysis.

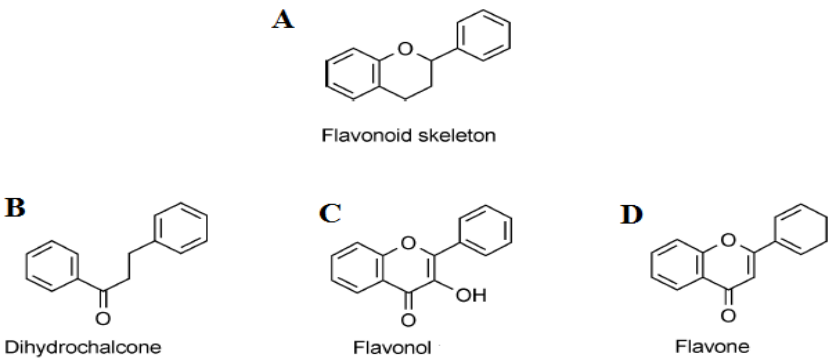


Figure 2.4. Structure of some flavonoids A) Basic flavonoid chemical structure, B) Dihydrochalcone chemical structure, C) flavonol chemical structure and, flavone chemical structure (Crozier et al. 2009).

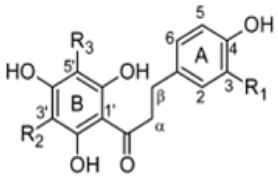
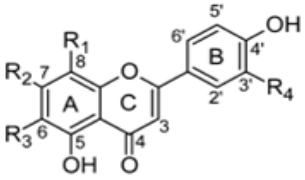
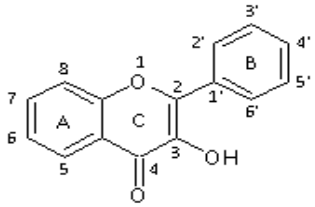
The major classes of flavonoids are flavanols (Figure 2.4C), flavones (Figure 2.4D), flavan-3-ols, anthocyanidins, flavanones and isoflavonones while the dihydrochalcones (Figure 2.4B) are the minor class (Appleton 2010). The paramount flavonoids in Rooibos are dihydrochalcones, flavones and flavanols (Joubert & de Beer 2011).

2.3. Flavonoid compounds in Rooibos

Rooibos tisane is a polyphenol rich infusion and important source of flavonoids. Flavonoids are significant and widely studied compounds in relation to health. They are of low toxicity in mammals when taken in recommended amounts (Appleton 2010). Rooibos contains three

prime classes of flavonoids as mentioned earlier in the previous section (Table 2.1); dihydrochalcones, flavanols, and flavones (Persson et al. 2010).

Table 2.1. Chemical structures of Rooibos flavonoids with ring labeling. A) dihydrochalcones, B) flavonols, C) flavones (Joubert, Beelders, et al. 2012).

Structure	Compound	Sustitution
A) Dihydrochalcones 	Nothofagin	R1=H, R2=C-β-D-glucosyl
	Aspalathin	R1=OH, R2=C-β-D-glucosyl
B) Flavones 	Orientin	R1=C-β-D-glucosyl, R2,R4= OH,R3=H
	Isoorientin	R1=H, R2,R4= OH,R3= C-β-D-glucosyl
	Vitexin	R1=C-β-D-glucosyl, R2= H,R3,R4=OH
	Isovitexin	R1,R4=H, R2= OH,R3= C-β-D-glucosyl
	Luteolin	R1,R3=H, R2=R4= OH
	Luteolin-*7-O*-glucoside	R1,R3=H, R2= *O*-β-D-glucosyl,R4= OH
	Chrysoeriol	R1,R3=H, R2= OH, R4=OCH_3
C) Flavonols 	Quercetin	R=OH
	Isoquercitrin	R= *O*-β-D-glucosyl
	Hyperoside	R= *O*-β-D-galactosyl
	Rutin	R= *O*-β-D-rutinosyl
	Quercetin-*3-O*-robinobioside	R= *O*-β-D-robinobiosyl

Flavonoid content varies with the quality of the Rooibos as higher quality grade contains higher levels of flavonoids (Joubert, et al. 2012). HPLC-DAD method was used to quantify the levels of flavonoids in the different grades of Rooibos by Joubert et al. (2012). A similar study was performed Beelders et al. (2012) to quantify the flavonoid content in aqueous extracts of unfermented and fermented Rooibos from two producers, Table 2.2.

Table 2.2. Phenolic mean content values in aqueous extracts of unfermented and fermented Rooibos plant material (Beelders et al. 2012).

Phenolic compound	Unfermented Rooibos (P1)	Unfermented Rooibos (P2)	Fermented Rooibos(P1)	Fermented Rooibos (P2)
PPAG	0.25 ± 0.051	0.44 ± 0.056	0.53 ± 0.10	0.60 ± 0.15
Isoorientin	0.72 ± 0.120	1.30 ± 0.160	1.30 ± 0.084	1.2 ± 0.16
Orientin	0.48 ± 0.080	0.81± 0.080	0.92 ± 0.053	0.92 ± 0.13
Aspalathin	8.40 ± 1.40	12.4 ± 1.000	0.64± 0.170	0.68 ± 0.190
Ferulic acid	nd	nd	0.09 ± 0.040	nd
Quercetin-3-*O*-rutinoside	0.43 ± 0.068	0.91± 0.120	0.94± 0.160	nd
Vitexin	0.093 ± 0.015	0.18 ± 0.017	0.19± 0.016	0.18 ± 0.013
Hyperoside	0.046 ± 0.0079	0.18 ± 0.046	0.26 ± 0.076	0.26 ± 0.073
Rutin	0.30 ± 0.047	0.42 ± 0.018	0.23 ± 0.140	1.2 ± 0.300
Isovitexin	0.13 ± 0.021	0.23 ± 0.029	0.20 ± 0.017	0.21± 0.014
isoquercitrin	0.090± 0.015	0.24± 0.038	0.18± 0.110	nd
Nothofagin	0.69 ± 0.120	1.7 ± 0.160	0.10 ± 0.026	0.071 ± 0.013

Content values expressed in g per 100g soluble solids ± standard deviation
P1= producer 1, P2= producer 2
nd= not detectable

Major flavonoids were identified in fermented Rooibos were aspalathin, isoorientin, orientin and also for the first time quercetin-*3*-*O*-robinobioside, an isomer of rutin. Quantitative analysis also showed that flavonoid content in Rooibos differs between producers (Beelders

et al. 2012). Heinrich et al. (2012) reported a 98 % decrease in aspalathin content during the fermentation of Rooibos which had been investigated and reported previously (Joubert & Villiers, 1997). Methanolic extracts as well as aqueous extracts unfermented and fermented Rooibos were analysed using the HPLC-DAD method during an investigation of Rooibos effects on glucocorticoid levels (Schloms et al. 2013). The data (personal communications), suggest that methanol and aqueous extracts had similar flavonoids levels. The aqueous extracts of unfermented Rooibos had higher concentrations of aspalathin and nothofagin than the aqueous extracts of fermented Rooibos, the same was observed in the methanolic extracts of unfermented and fermented Rooibos. Comparison of methanolic extracts and aqueous extracts of Rooibos showed that methanolic extracts contained higher levels of flavonoids than the aqueous extracts. Methanolic extracts of Rooibos were used to investigate further the effects of Rooibos on steroidogenesis in the current study. The extracts were quantified using the HPLC-DAD method that was used by Beelders et al. (2012) and Schloms et al. (2013), consequently the results will be discussed in Chapter 4 and Chapter 5. The effects of the methanolic extracts on steroidogenesis are discussed in Chapter 4 and Chapter 5 as well. The major flavonoids found in Rooibos are:

Dihydrochalcones: Aspalathin and nothofagin are the dihydrochalcones found in Rooibos. Rooibos is the only source of aspalathin whereas nothofagin has other sources that include the plant *Nothofagus fusca* (Joubert 1996). Asphalathin has been reported to have higher anti-oxidant and anti-mutagenic activities than other flavonoids. This adds to the novelty of Rooibos herbal infusion (de Beer et al. 2012). During fermentation, aspalathin is broken down to its flavone analogues; isoorientin and orientin as shown in Figure 2.5. Nothofagin, a 3-deoxy analogue of aspalathin, is present in lower quantities in Rooibos than aspalathin. Nothofagin's flavone analogues comprise of regioisomers, vitexin and isovitexin. It is known to be a good radical scavenger but cannot protect cell membranes from peroxidation (Snijman et al. 2009). The dihydrochalcones are unstable and their concentration decreases significantly during fermentation (Joubert, de Beer, et al. 2012)

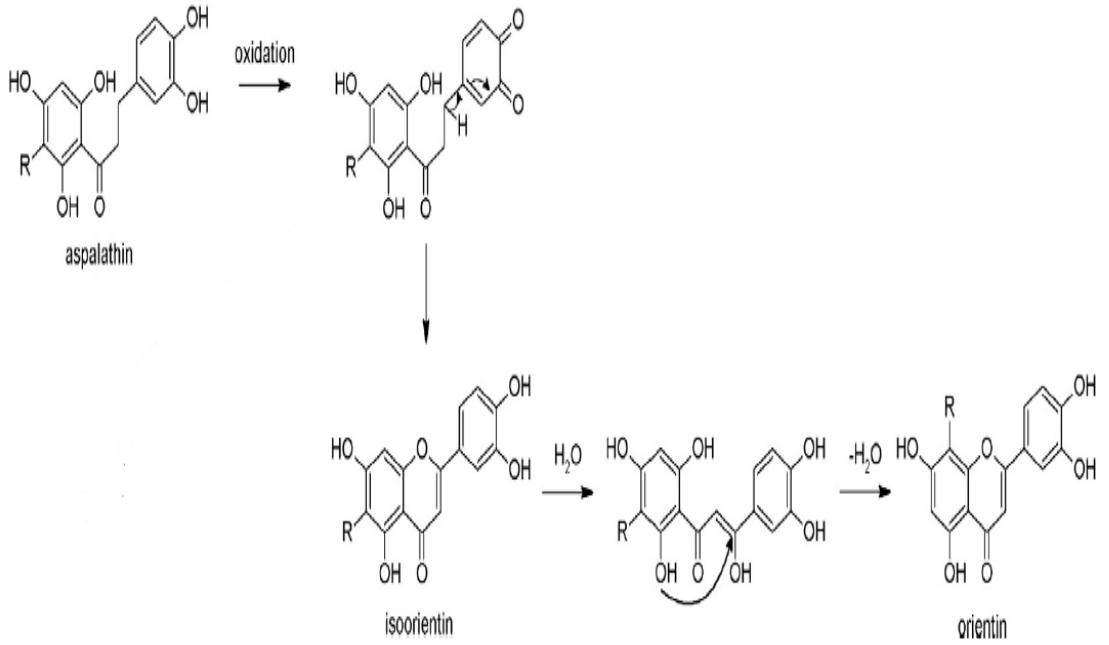


Figure 2.5. Modified pathway of aspalathin oxidation during fermentation of Rooibos (Joubert et al. 2011)**.**

Flavanols: Flavonols are yellow in colour and are found in many plants. There are a number of flavonols found in Rooibos as shown in Table 2.2 but only rutin and quercetin are going to be discussed. Unlike other flavonols, rutin and quercetin are used as dietary supplements as they are found in many medicinal plants such as horse chestnuts, ginkgo and many others. Rutin, is abundant in many plants and is a flavonol glycoside used to decrease capillary fragility (Raymond 2009). Quercetin is the one of the most abundant and common flavonoid that has been extensively investigated. In human diets and plants, quercetin is present in glycosylated forms such as quercetin-*3*-*O*- rutinoside (rutin) and isoquercitrin (Vrba et al. 2012). It has anti-oxidant activities and it increases absorption of vitamin C in mammals (Appleton 2010). Quercetin has been reported to have analgesic properties (Filho et al. 2008).

Flavones and isoflavones: These compounds are pale yellow, they exist as coloring agents in plants (Rice-Evans et al. 1996). They are structurally similar to flavonols but lack oxygenation at carbon 3. Rooibos contains low amounts of apigenin-8-C-glucosides (vitexin and isovitexin). Flavone and isoflavones are structurally similar to eostrogen thus are classified as phytoestrogens (Del Rio et al. 2013). Phytoestrogens are non-steroidal constituents of our diets and act as antagonists or agonists of estrogen receptors. They control the activities of the key enzymes in estrogen biosynthesis through binding to the receptor

(Brozic et al. 2006). These compounds play an important role in cancer prevention as they are associated with reduced cancer rates (Birt et al. 2001).

Processing of Rooibos to produce fermented Rooibos, affects the flavonoid content adversely. The ratio of dihydrochalcones and flavonoids in Rooibos decreases with the fermenting process (Standley et al. 2001). As mentioned previously in this section, during fermentation, aspalathin is broken down or oxidised to flavones (isoorientin and orientin) thus the concentration of aspalathin reduces whilst that of flavones increases (see Figure 2.5), (Joubert & de Beer 2011). Not only is the flavonoid content affected by fermentation but also the colour and aroma of Rooibos. Heinrich et al. (2012) reported that the colour change of Rooibos during fermentation is based on non-enzymatic mechanisms of aspalathin degradation (Heinrich et al. 2012). Nothofagin has been reported to be structurally similar to aspalathin thus also undergoes oxidation during processing of Rooibos (Bramati et al. 2002).

2.4. Bioavailability of flavonoids

The biological properties of Rooibos and its flavonoids in the mammalian body depend on their bioavailability. Bioavailability is the degree to which a drug or a substance becomes available to the target tissue. Evidence of a few phenolic compounds bioavailability has been obtained by quantifying or identifying their concentrations in blood plasma and urine. The bioavailability is determined after ingestion of known and quantified food content or ingestion of quantified pure flavonoids (Scalbert & Williamson 2000). Distribution and bioavailability in tissues and organs of each phenolic compound may be affected by its physical properties which include their differences in solubility, molecular size and polarity (Liu et al. 2004). However, bioavailability of polyphenols is affected by a number of factors (see Table 2.3) (D'Archivio et al. 2010).

Table 2.3. Factors that affect bioavailability of flavonoids (D'Archivio et al. 2010)

External factors	Environmental factor (that is, sun exposure, degree of ripeness); food availability
Food related factors	Food matrix; presence of positive or negative effectors of absorption (that is, fat fibre)
Food processing related factors	Thermal treatments ; lyphylisation; methods of culinary preparation; cooking; homogenisation; storage; cooking
Interaction with other foods	Bonds with proteins(that is, albumin) or with polyphenols with similar mechanism of absorption
Polyphenols related factors	Chemical structure; concentration in food; amount introduced
Host related factors	Intestinal factors (that is, enzyme activity; intestinal transit time; colonic microflora) Systematic factors (that is, disorders; pathologies; gender and age; genetics; physiological conditions

2.5. Absorption and metabolism of flavonoids

Little is known about absorption and metabolism of polyphenols and it is likely that different classes of flavonoids have different pharmacokinetics properties (Rice-Evans et al. 1996). The biological fate of the flavonoids in the human diets is complex and depends on a number of processes. It is believed that flavonoids cannot be absorbed after ingestion but are first hydrolysed to their aglycones by bacterial enzymes found in the intestines. There is evidence that flavonoids are absorbed along the gut but the mechanism has not been established yet. There are suggestions that the hydrolysed simpler molecules of flavonoids are absorbed through passive diffusion and sodium dependent glucose transporter 1 (Walle 2004). It is hypothesised that flavonoids are absorbed by passive diffusion after the conversion of

glycosylated flavonoids to aglycones by the colon microorganisms. After absorption, the flavonoids are metabolised to phenolic acids or conjugated compounds in the liver by sulfation or methylation (Yao et al. 2004).

It has been apparent that hydrolysis of flavonoids starts in the oral cavity then aglycones are absorbed in stomach by passive diffusion and the glycosides are absorbed in the intestines. Only a small concentration of flavonoids is absorbed and most of the flavonoids ingested are metabolised to simpler components by the microflora in the colon and are subsequently absorbed (Raymond 2009). Bacterial hydrolysis of flavonoids occurs in the colon to a greater extent, and to a lesser extent in the oral cavity and small intestines (Saura-Calixto et al. 2007). Recent reports were made by Del Rio et al. (2013) that some flavonoids diffuse through the small intestines into the blood but others, such as the flavonoid glycosides are cleaved to release their aglycone by lactase phloridzin hydrolase. The aglycone consequently diffuses through the walls of the intestine as a result of its increased lipophilicity and proximity to the cell membranes. Active sodium dependent glucose transporters 1 are involved in the transportation of the flavonoids through the small intestine. However these flavonoids and their aglycones are capable of inhibiting transporters in the small intestine (Del Rio et al. 2013).

Metabolism of quercetin glycosides (rutin) starts in the small intestine primarily where the sugar moiety is removed by enzymatic reactions. The released quercetin may be converted to taxifolin by enteric bacteria or degraded to phenolic acids if not absorbed (Vrba et al. 2012). *In vivo* studies have shown that aspalathin is absorbed in the gut and undergoes bacterial action in the intestines forming free aglycones after oral administration. Seven and eleven days later, methylated aspalathin was found in the urine but not in plasma (Kreuz et al. 2008). Aspalathin and nothofagin bioavailability in human urine was confirmed by Breiter et al. (2011) as well as the methylated aspalathin which was the main excreted metabolite (Breiter et al. 2011).

The form of the flavonoid seem to influence the rate of absorption as it has been suggested that quercetin (glucosylated form) is absorbed in significant amounts (Nijveldt et al. 2001). Moreover, absorption of flavonoids is influenced by other factors that include the molecular weight, size and the extent and type of esterification (Scalbert et al. 2002). Del Rio et al.

(2013) illustrated Metabolism of rutin to quercetin by colon enzymes where the sugar moieties are cleaved off to produce quercetin (Figure 2.6).

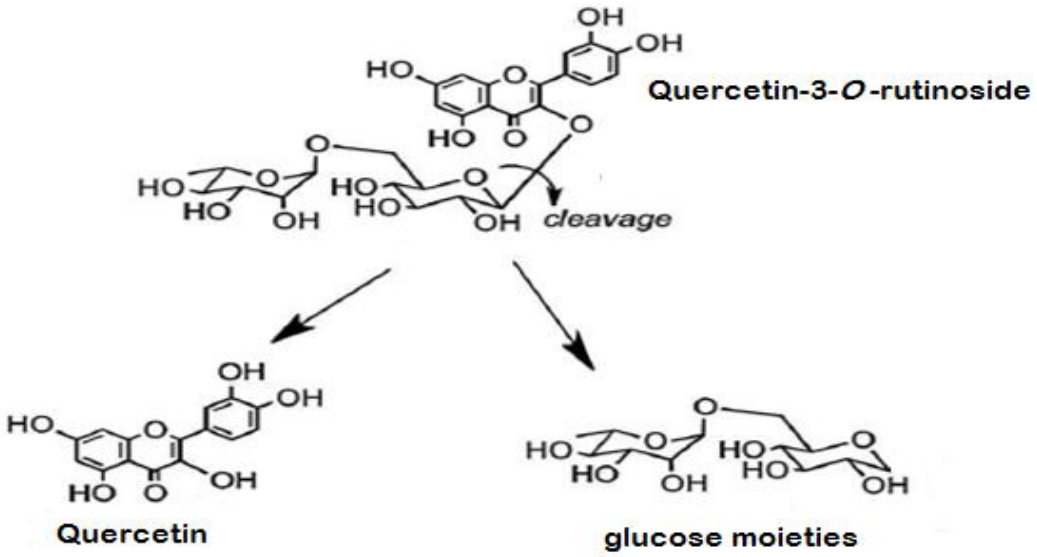


Figure 2.6. Proposed quercetin-*3-O*-rutinoside bacteria catabolism pathway of in the colony of humans (Del Rio et al. 2013).

Flavonoids have the ability to bind to serum proteins (Del Rio et al. 2013). Albumin is the prominent serum protein in blood responsible for binding of flavonoids. The degree to which flavonoid binds to albumin affects the rate of delivery to tissues and cells in the body as cellular flavonoid uptake is proportional to the unbound flavonoids (Xiao et al. 2012). Absorbed flavonoids are transported to the liver bound to albumin proteins to undergo phase II biotransformation or catabolised to simpler phenolic compounds before excretion in urine or bile (Cabrera et al. 2006; de Mejia et al. 2009). Phase II biotransformations usually occur in the colon and liver. In the liver, the flavonoids undergo sulfination or methylation and glucoronidation during biotransformation (Villaño et al. 2010). However the biotransformed flavonoids are converted to polar conjugates that can be easily excreted thus affecting the bioavailability of flavonoids (Raymond 2009). Methylated, sulphated and glucuronidated metabolites were found in human urine by Stalmach et al. (2009) during *in vivo* studies (Stalmach et al. 2009).

2.6. Beneficial health effects of Rooibos and its flavonoids

Anecdotal reports suggest that the Khoisan were aware of the medicinal properties of Rooibos as they used it as a remedy to calm the nervous and digestive system, and to treat insomnia, allergies and skin diseases (Van Niekerk & Viljoen 2008). It has been widely reported that these characteristic health benefits of plant based foods and beverages are attributed by phytochemicals in plants, particularly Rooibos (Snijman et al. 2007). Some of the phytochemicals are available on the market some over the counter such as rutin and quercetin, just to name a few. Rutin is medically used in topical cream or is ingested for the maintenance of blood capillary integrity. Flavanols (rutin and quercetin) have been reported to have chemopreventative, cardioprotective, neuroprotective, anti-inflammatory and anti-allergic activities (Appleton 2010).

Rooibos has also been used as a milk substitute to treat colic in infants. Annekie Theron, a South African woman, discovered that the Rooibos infusion would ease her infant's colic and since then, Rooibos has been recommended for the treatment of colic (Van Wyk 2011). Since Rooibos doesn't contain caffeine and has low levels of tannins, it is safe for pregnant women to take as it will not cause them any discomfort and their blood iron levels will not be depleted (Council 2013). The consumption of Rooibos has been proven not to have any side effects. Marnewick et al. (2003) found that consumption of Rooibos tea by rats for a period of ten weeks did not result in adverse effects in either the liver or kidney. In a human study conducted by Marnewick et al. (2011), it was noted that upon consumption of six cups of Rooibos per day for six weeks, no side effects were reported.

In vivo experiments on iron absorption were conducted on a group of volunteers administered elemental iron (16 mg) in the presence of Rooibos, black tea and water respectively. The amount of haemoglobin, ferritin and transferrin in blood plasma was measured after ingestion of iron and the results indicated that the mean iron absorption in the presence of Rooibos (7.25 %) or water (9.34 %) were similar but for the black tea (1.70 %) was much lower. The differences of iron absorption capacity between Rooibos and black tea were probably due to the lower levels in tannin in Rooibos (Heeseling et al. 1979). In the cosmetic industry, Rooibos has also been shown to reduce wrinkles to a greater extent than other teas (Chuarienthong et al. 2010). Rooibos was also shown to improve hair growth and condition.

Last but not least, it has been shown that Rooibos can be used as after sun lotion as it soothes the skin. The skin is the largest part of the body and is exposed to environmental oxidative stress, thus the introduction of Rooibos in topical cosmetic formulations become popular to reduce photo-aging and ultraviolet radiation damage (Mavon et al. 2004). These characteristics of Rooibos are believed to be related to its anti-microbial, anti-oxidant and anti-inflammatory properties (Tiedtke & Marks 2002).

Recent studies have shown that Rooibos' biological activities include antioxidant, anti-mutagenic, anti-cancer, anti-viral, anti-fungal, and anti-stress activity (Sarwar & Lockwood 2010). Anti-oxidants are responsible for inhibiting oxidant formation or intercepting oxidants or repairing oxidant damaged cells or neutralising the oxidants by donating one of their electrons to the free radical. Oxidants are metabolites that cause oxidative stress which is a condition in which oxidant metabolites (free radicals) become toxic to the body. The condition could be due to overproduction of the free radicals or a change in protective cellular mechanisms (Kaur & Kapoor 2001; Erguder et al. 2007). Lifestyle, environment, and pathological conditions can result in excess oxidative stress which affects normal cellular processes leading to chronic diseases (Willcox et al. 2004). This condition damages the DNA, important proteins and cell membranes which eventually lead to cell death (Valko et al. 2006). Studies have shown that both unfermented and fermented Rooibos exhibit anti-oxidant activities with unfermented Rooibos having a much higher activity than the fermented Rooibos (Joubert et al. 2008). The difference in the anti-oxidant activity is due to the oxidation of flavonoids during fermentation of Rooibos hence the change in flavonoid content (Joubert et al. 2011).

Polyphenols have been shown to be more effective anti-oxidants *in vitro* than vitamin C and vitamin E because of their chemical structure which is ideal for free radical scavenging activity. Flavonoids have been documented to act as free-radical scavengers (anti-oxidants) because of their reducing properties as electron or hydrogen-donating agents (Rice-Evans et al. 1996). *In vivo* studies done by Marnewik et al. (2003) have shown that the flavonoids found in Rooibos mimic the activity of a α-tocophenol, a fat soluble anti-oxidant compound, thus act as anti-oxidants (Marnewick et al. 2003). Some flavonoids have been reported to exert their anti-oxidant activity by interacting with the cell signalling and modulate cell activities (Hatti et al. 2009). Their anti-oxidant activity is exerted by the flavonoids, either by

scavenging the free radicals or inhibiting the activity of enzymes that generate free radicals (Nijveldt et al. 2001). Moreover, Oh et al. (2013) reported that anti-ooxidant activity of flavonoids usually occurs through redox mechanisms and allows the flavonoids to act as metal chelators, hydrogen donors, reducing agents and singlet oxygen quenchers.

Most *in vitro* studies done by various investigators have shown that the unfermented Rooibos has a higher anti-oxidant capacity than the fermented Rooibos as previously mentioned. In a another study done by Joubert et al. (2004) where the scavenging capacity of flavonoids found in Rooibos was tested on oxidative radicals (DPPH and O_2^-), it was observed that the flavonoids asphalathin, quercetin, orientin and luteolin have anti-oxidant activities, though aspalathin and quercetin showed more anti-oxidant activity than the other flavonoids. Anti-oxidant activity of Rooibos in cellular systems was observed in several investigations when roughly 20 µg/mL of the extract was used. The Rooibos concentration at cellular levels inhibited the generation of free radicals (McKay & Blumberg 2007).

Soluble extracts of unfermented and fermented Rooibos extracts have inhibited lipid peroxidation in the presence of iron in liver microsomal preparation. In this investigation, formation of reactive substances that caused lipid peroxidation was measured (Marnewick et al. 2005). It has been reported that flavonoids are oxidised to quinones and dihydrochalcones after quenching radicals and that the new compounds exhibit higher anti-oxidant activity than the other flavonoids (flavones and flavonols) in Rooibos tea (Krafczyk et al. 2009).

Anti-viral activity investigations of Rooibos have been done in the context of HIV. An investigation by Nakano et al. (1997) in infected MT-4 cells in the presence of acid polyssacharides extracted from Rooibos showed that the cytopathicity in HIV was suppressed. The suppression of the cytopathicity was shown by the inhibition of HIV virus binding to the MT-4 cells at a concentration of 25 µg/mL of Rooibos. This study showed the anti-viral activity of chemically extracted polyssacharides, but these polysaccharides have subsequently been reported not to be found in the Rooibos infusion made by steeping the leaves in hot water, (Erickson 2002). The unavailability of the polysaccharides in aqueous Rooibos regards the reported anti-viral activity of Rooibos not valid; thus more investigations need to be conducted in regard to its anti-viral activity.

In another study conducted in Japan, it was shown that concentrations ranging from 1 µg/mL to 100 µg/mL of Rooibos extract induced the production of antigen specific antibodies with an increase in interleukin-2 production in murine splenocytes (Kunishiro et al. 2001). Interleukin-2, which is normally produced during immune response to in the body by T-cells, is important for growth, proliferation and differentiation of T-cells (Waldmann 2006). There is still more studies to be done regarding the effects of Rooibos' immunological properties.

Anti-mutagenic properties of Rooibos against direct acting mutagens, (2 acetylaminofluorene and aflaxtoxin) that required metabolic activation, in the presence of aqueous Rooibos were investigated. A *Salmonella typhimurium* mutagenic assay was used and it was observed that mutagenesis was suppressed in the presence of Rooibos (Marnewick et al. 2000). It was suggested that the Rooibos inhibitory effects result from the interaction of the P450 enzyme responsible for the mutagen activation and the different constituents of the Rooibos extract. The Rooibos extract constituents are polyphenols which act as acceptors of electrons from NADPH from P450 enzyme mediated reactions; thus activation of the mutagens is suppressed. Moreover, it was suggested that the structure of flavonols allow the flavonols to act as nucleophilic centres enabling them to form flavonol-carcinogen adducts with electrophilic carcinogens thus preventing tumorigenesis (Marnewick et al. 2000). Marnewick colleborated with other scientists such as Sissing to investigate the effect of aqueous Rooibos on methylbenzylnitrosamine (a carcinogen) induced oesophageal cancer cells and they observed that there was a reduction in the mean total papilomma size. The reduction was noted due to the absence of large papilomas (>10 mm^3) (Sissing et al. 2011). It has been reported that skin 12-*O*-tetra-decanoylphorbol-13-acetate (TPA) induced tumours on mice's skin were reduced in the presence of topically applied fermented Rooibos extract, but in the presence of unfermented Rooibos extract, no tumours were observed (Marnewick et al. 2005).

A review of investigations of *in vivo* and *in vitro* effects of Rooibos in animal and humans was documented by Mckay et al. (2007) suggested that Rooibos has anti-mutagenic and anti-cancer properties. One study done by Komatsu et al. (1994) observed that oncogenic transformation of mouse embryo cells induced by X-ray was suppressed as cell survival decreased in the presence of increasing concentrations of Rooibos. Lamosova et al. (2007) showed that proliferation and growth of muscle cells of the chick embryo was inhibited in a

dose-dependent manner of Rooibos extract. The inhibition was viewed from the decrease in DNA content in the presence of Rooibos.

Luteolin and quercetin have been observed to induce apoptosis and reduce proliferation of thyroid and colon cancers respectively *in vitro*. It is not known whether these anti-cancer activities of these flavonols can occur *in vivo* due to their low concentrations in Rooibos (Mori et al. 2001). Orientin was shown to inhibit oxidative degradation of lipids, thus preventing cancer (Vrinda & Devi 2001). More studies are required to determine the mechanisms by which Rooibos exerts its anti-cancer properties.

Diabetes is a condition in which an individual (usually mammals) has high blood glucose levels. The ceasing of insulin production or irresponsiveness of cells to insulin causes the high blood glucose levels. Untreated diabetes can lead to complications such as cardiovascular complications, retinal damage, miscarriages and renal failure (Fiorino et al. 2012). It is also a lifestyle related chronic disease and treatment has been linked to regular intake of polyphenols which maintain redox homeostasis and cell signalling (Coetzee et al. 2013). Aqueous Rooibos extract has been shown to have anti-diabetic properties as it lowered plasma triacylglycerols and cholesterol, plasma urea, creatinine and aminotransferase concentrations when compared with untreated diabetic rats (Ulicná et al. 2006). Aspalathin, a dihydrochalcone was reported to significantly reduce hyperglycemia in a dose-dependent manner. In addition to hyperglycemia reduction, aspalathin was reported to reduce glucose intolerance in mice by increasing glucose uptake and insulin secretion. The aspalathin was purified from unfermented Rooibos and concentrations ranging from 1- 100 µM were used to determine its effects on insulin secretion from cultured pancreatic cells and cultured muscle cells *in vitro*, and fasting blood glucose of type 2 model mice *in vivo* (Kawano et al. 2009).

Recent studies have shown that polyphenols have the ability to reduce the occurrence of diabetes by preventing pancreatic cell function disorder. Polyphenols have been reported to influence glucose metabolism through several mechanisms such as modulating hepatic glucose output, inhibiting carbohydrate digestion and glucose absorption in the intestine, stimulating secretion of insulin from the pancreatic cells, modulating glucose release from the liver and activation of insulin receptors which leads to glucose uptake in insulin-sensitive tissues (Hanhineva et al. 2010; Thomas & Pfeiffer 2012). Rutin has anti-diabetic properties as

it decreases blood glucose levels in streptozotocin induced diabetic rats by inhibiting α-glucosidases enzymes that break down starch and disaccharides to glucose (Muller et al. 2012). Sanderson et al. (2013), observed inhibition of lipid accumulation in mouse embryonic fibroblasts by 22 % and 15 % with 10 µg/mL and 100 µg/mL respectively of aqueous Rooibos soluble solids. The group also noticed an increase in intracellular ATP concentrations in the cells which are associated with increased uptake of glucose. This study suggest the Rooibos soluble solids' has potential in preventing obesity as it showed Rooibos solids to affect adipocyte metabolism and inhibit adipogenesis (Sanderson et al. 2013).

Hypertension (high blood pressure), also known as the silent killer, is a condition that is usually underdiagnosed or inadequately treated. Most people with this condition are not conscious of this disease till they have their blood pressure taken. Hypertension is a major risk of chronic diseases such as cardiovascular and renal diseases (Steyn 2005). Clinical trials have demonstrated the benefits of controlling blood pressure. High blood pressure is usually treated by beta blockers or angiotensin converting enzyme (ACE) inhibitors amongst others but these treatments have adverse effects (Tedla et al. 2011). It is usually associated with mineralocorticoid excess (Blumenfeld & Sealey 1994), a steroid hormone that will be discussed in Chapter 3.

Anecdotally Rooibos has been used as treatment of cardiac arrhythmias and hypertension. In a study done by Khan et al. (2006), it was shown that the aqueous extract of Rooibos had a smooth muscle relaxing effect that could have been mediated through activation of potassium channels and calcium antagonist mechanisms. Potassium channels play important roles in cardiac repolarisation, smooth muscle relaxation and insulin release, which are involved in regulation of physiological functions (Sandhiya & Dkhar 2009). Persson et al. (2006) has observed an increase in nitric oxide concentration in human endothelial cells when incubated with aqueous Rooibos extract. Nitric oxide inhibits ACE activity which is a key enzyme in the renin-angiotensin-aldosterone system (RAAS). RAAS is important for the regulation of blood pressure, electrolyte and fluid balance (Persson et al. 2006). The RAAS will be discussed in Chapter 3. In a cardiovascular *in vivo* study where 20 volunteers drank 400 mL of aqueous Rooibos, it was observed that Rooibos significantly inhibited ACE activity (Persson et al. 2010). The mechanism by which Rooibos inhibits the ACE activity was

suggested to be a mixed type of inhibition involving non-competitive and competitive inhibition (Persson 2012).

It has also been observed that flavonoids can inhibit and sometimes induce a large variety of enzyme activity in mammals (Hollman & Katan 1999). Flavonoids have been proven to be steroidogenesis modulators (Hatti et al. 2009). Some flavonoids have been reported to modulate steroid metabolism by altering the activity of P450 enzymes responsible for steroid glucuronidation, sulfonation and hydroxylation (Cheng & Li 2012). Schloms et al. (2012) observed that unfermented Rooibos methanol extract inhibited the activity of some cytochrome P450 enzymes, e.g, CYP 21-hydroxylase (CYP21A2) and CYP 17-hydroxylase/17,20 lyase (CYP17A1). These enzymes are key enzymes in the biosynthesis of steroids particularly aldosterone (ALDO), a mineralocorticoid and its precusors, DOC and corticosterone (CORT) which are glucocorticoids (Schloms et al. 2012).

The inhibitory effect of unfermented Rooibos methanol extract was observed in steroidogenic human adrenal carcinoma cells (H295R). It was observed that the total amounts of steroids decreased significantly in basal and forskolin stimulated cells in the presence of the unfermented Rooibos methanol extract. Moreover it was observed that ALDO and its precursors concentration was significantly reduced under forskolin conditions in the presence of Rooibos extracts (Schloms et al. 2012). Forskolin is a steroidogenesis stimulant as it mimics adrenocorticotrophic hormone (ACTH), to which the H295R cell line is not responsive. The HPA axis and RAAS will be discussed in Chapter 3. Dihydrochalcones flavonoids (aspalathin and nothofagin) have been shown to contribute to the alleviation of symptoms of adrenal steroidogenesis disorder. Their effects were shown in the reduction of total steroids produced by H295R cells under stimulated conditions in the presence of these flavonoids. Some enzymes' activities of the adrenal steroidogenesis system (CYP17A1 and CYP21A2) were inhibited by these flavonoids (Schloms et al. 2012).

2.7. Interaction of flavonoids with P450 enzymes

Cytochrome P450 enzymes are a functionally diverse group of heme proteins with broad substrate specificity. The enzymes are involved in the oxidation of exogenous and endogenous organic substances that include xenobiotics, steroids and lipids (Seden et al. 2010). P450 enzymes also sustain homeostasis in mammals as they produce steroid hormones

that maintain homeostasis. It has been reported that these enzymes interact with flavonoids in three ways (Helene 2009); either by being inhibited or stimulated by flavonoids, or their biosynthesis is induced by the flavonoids or some P450 enzymes metabolise the flavonoids.

Ohno et al. (2002) reported inhibition of H295R microsomal P450 enzymes by some flavonoids, particularly flavones and isoflavones. In this study, Ohno et al. (2002) described the inhibition to be a result of the hydroxyl group on the 6th position of the pyran ring or the 4th position of the benzene ring of the flavonoids. Inhibition of these enzymes may aid in the decrease of basal concentrations of mineralocorticoids and glucocorticoids thus decreasing the risk of chronic diseases. In a recent study, it has been shown that non-hydroxylated flavones upregulate the expression of CYP11B1 causing the increase in cortisol production in H295R cells. This upregulation of this enzyme has profound adverse consequences that include high basal cortisol levels (Cheng & Li 2012).

Since flavonoids are ubiquitous to plants, Platt et al. (2010) did a recent study on P450 enzymes and several plants. Some cytochrome P450 enzymes expressed in genetically engineered Chinese Hamster cells were shown to be inhibited by Rooibos, green tea, some fruits and some vegetables. This inhibition aids in decreasing risks for cancer (Platt et al. 2010). Continuous ingestion of herbal infusions, particularly Rooibos and guava, showed that it induced the biosynthesis of a very important intestinal P450 enzyme (CYP3A). CYP3A is involved in the metabolism of several important prescription drugs such as HIV protease inhibitors and calcium channel blockers (Matsuda et al. 2007). Hence, inhibition or low concentration of this enzyme may lead to blood and tissue toxicity. Vrba et al. (2012) recently reported flavonoid (quercetin) induction of P450 enzyme (CYP1A1) expression in *in vivo* experiments. According to Vrba's report, there was an increase in the expression of CYP1A1 which metabolises drugs and is involved in the activation of pro-carcinogens (Vrba et al. 2012).

2.8. Conclusion

Rooibos has risen to worldwide popularity as it is a polyphenol rich herbal infusion containing low concentrations of tannin, known to be caffeine free. Not only is Rooibos popular, it has created a market in South Africa thus boosting the economics of the country by creating jobs. The herbal infusion contains high concentrations of anti-oxidants and is safe

to drink with no reported side effects or toxic effects as discussed in the previous sections. Rooibos herbal infusion is suggested to be a good option for those who like to drink mild, non-stimulating, or sedating beverages. Rooibos is processed to make two different kinds of Rooibos, the fermented and the unfermented Rooibos which are different in the way they are processed, taste and flavonoid content. The unfermented Rooibos is unique to other herbal infusions such as the black and green tea as it has a unique brick red colour accompanied with a sweet fruity taste. Unfermented Rooibos has higher levels of polyphenols than fermented Rooibos and thus is considered healthier than the fermented Rooibos.

In vivo and *in vitro* studies done on Rooibos have corroborated some of the anecdotal reports about this herbal drink. Rooibos herbal drink's health properties include alleviation of symptoms of the digestive system, respiratory system, endocrine system, skin diseases, sleeplessness, and stress. Some investigations show that Rooibos affects some of the human body homeostasis mechanisms. Homeostasis mechanisms include the HPA axis, the RAAS and their elements such as the enzymes, in particular some of the P450 enzymes. The HPA axis and the RAAS will be discussed in the following chapter.

In the chapter 4 and 5, there are investigations and discussions on the effects of Rooibos on steroidogenesis and P450 enzymes. These Chapters will provide more information in which type of Rooibos is more effective on affecting some of the key enzymes in the mineralocorticoid and glucocorticoid pathways of steroidogenesis. Also the investigations will provide scientific evidence for its proposed capacity to alleviate hypertension.

CHAPTER 3

Adrenal steroidogenesis and cytochrome P450 enzymes

3.1. Introduction

Homeostasis is the body's ability to maintain and regulate its inner physiological environment in response to various stimuli. Stimuli, which influence homeostatsis can be physical, mental, or environmental. Internal or external stimuli, also referred to as stressors, can cause adverse effects on the regulation of physiological activities. The two main physiological systems in mammals that regulate and control homeostasis are the central nervous system and the endocrine system. Several organs in the human body, including the hypothalamus, pituitary, pancreas, thyroid, kidney, adrenal glands and gonads are involved in the maintenance of homeostasis. The brain maintains homeostasis through its control of the central nervous system, while the endocrine glands control homeostasis through the secretion of specific hormones. The adrenals and gonads specifically synthesise and secrete steroid hormones. Steroid hormones are communicative substances responsible for the maintenance of the reproductive processes, mineral balance and stress tolerance.

Control and maintenance of steroid biosynthesis, also known as steroidogenesis, is important as it maintains and regulates a wide range of physiological functions. Changes in steroid levels in the body activate compensatory mechanisms to restore normal physiological functions as well as normal steroid concentrations. Steroid hormone imbalances lead to clinical conditions which include type 2 diabetes, cardiovascular diseases, metabolic syndrome, insulin resistance, renal failure, and hypertension. The regulatory mechanisms for steroid hormone biosynthesis include the hypothalamic-adrenal-axis (HPA-axis) and renin-angiotensin-aldosterone-system (RAAS). The HPA-axis is activated by the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus, which activates secretion of adrenocorticotropic hormone (ATCH) from the pituitary gland into the bloodstream. ACTH induces steroidogenesis, particularly glucocorticoid synthesis and secretion (Pariante & Lightman 2008), by binding to a receptor located in the adrenal. The RAAS is activated by the secretion of renin from the kidneys, which activates angiotensin II (Ang II) from angiotensinogen, which is secreted from the

liver. Ang II finally induces mineralocorticoid synthesis and secretion from the adrenals (Atlas 2007). These two systems are further discussed in section 3.7 of this Chapter.

In the adrenal gland, more specifically in the adrenal cortex steroid production is catalysed by the steroidogenic enzymes, which include cytochrome P450-dependent and hydroxysteroid isomerase-dehydrogenase enzymes. Cholesterol, the initial substrate for adrenal steroidogenesis, undergoes a series of hydroxylation and lyase reactions to produce glucocorticoids, mineralocorticoids and adrenal androgens (Shimozawa et al. 1993). The plasma levels of these steroids are regulated by a negative feedback inhibition system on the HPA-axis and RAAS (Payne & Hales 2004). A dysfunctional HPA-axis and RAAS can lead to depression, stress, anxiety, cardiac diseases, kidney diseases, and diabetes. The concentration of corticosteroids such as cortisol (a glucocorticoid) and ALDO (a mineralocorticoid), can be measured in the blood and can serve as biomarkers for adrenal diseases (Stewart et al. 1993).

3.2. Adrenal gland anatomy and zones of steroidogenesis

Adrenal glands are endocrine encapsulated bilateral glands situated on top of each kidney. The glands are comprised of two functionally different tissue types that produce different types of hormones. The inner medulla (neurological tissue) is responsible for catecholamine production such as adrenaline (epinephrine) and norepinephrine while the outer cortex (glandular tissue), which forms the largest part of the adrenal, is responsible for steroid hormone biosynthesis (Engeland et al. 1997).

The adrenal cortex is divided into three zones, the zona glomerulosa, zona fasciculata and the zona reticularis (see Figure 3.1). The selective expression of unique steroidogenic enzymes in each zone determines the unique steroids produced. The zona glomerulosa is the outermost zone which expresses enzymes responsible for the biosynthesis of mineralocorticoids. The zona fasciculata is the middle zone that expresses enzymes responsible for the biosynthesis of glucocorticoids. The zona reticularis expresses enzymes that catalyse the biosynthesis of androgens (sex hormones) (Rainey 1999; Rainey et al. 2002).

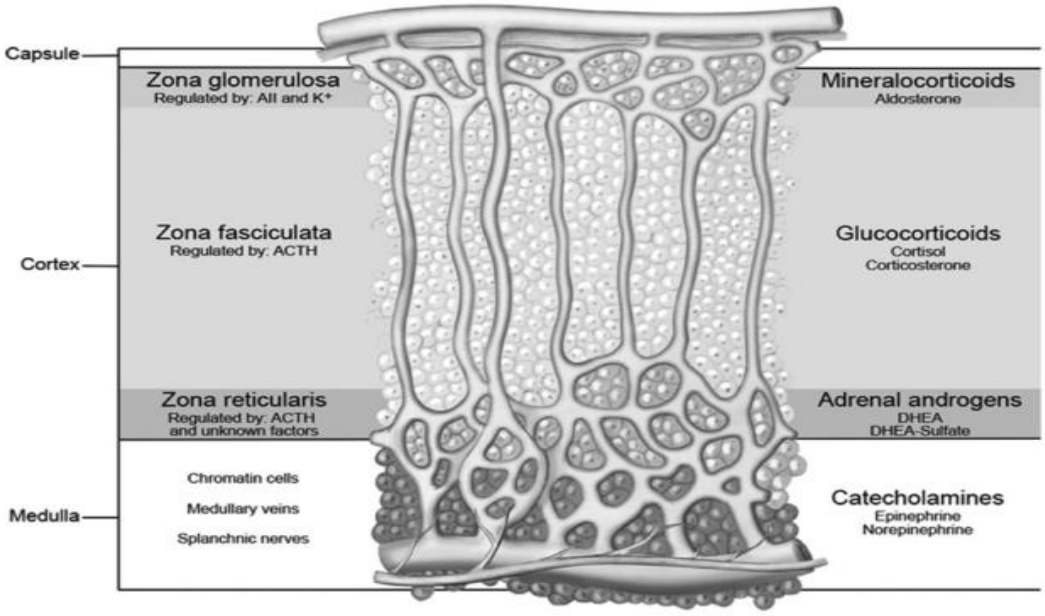


Figure 3.1. Functionally distinct zones of the adrenal cortex. The zona glomerulosa secretes mineralocorticoids, the zona fasciculata secretes cortisol and the zona reticularis secretes adrenal androgens (Wang & Rainey 2012).

Each steroidogenic adrenal zone has its own distinct steroidogenic pathway, see Figure 3.2. The initial substrate for steroidogenesis, cholesterol, is converted to pregnenolone (PREG) in all the adrenal zones. The zona glomerulosa is distinguished from the other zones as it does not express CYP17A1 thus there is no production of cortisol or androgens (see Figure 3.2A). CYP17A1 is expressed in both zona fasciculata and zona reticularis, producing cortisol and androgens. CYP11B2 is expressed in the zona glomerulosa, which is important for the final steps in ALDO biosynthesis. Moreover, the zona glomerulosa expresses Ang II receptors (Slominski et al. 1999). Ang II is a hormone that stimulates ALDO biosynthesis and is under regulation of the RAAS which is discussed in section 3.7.

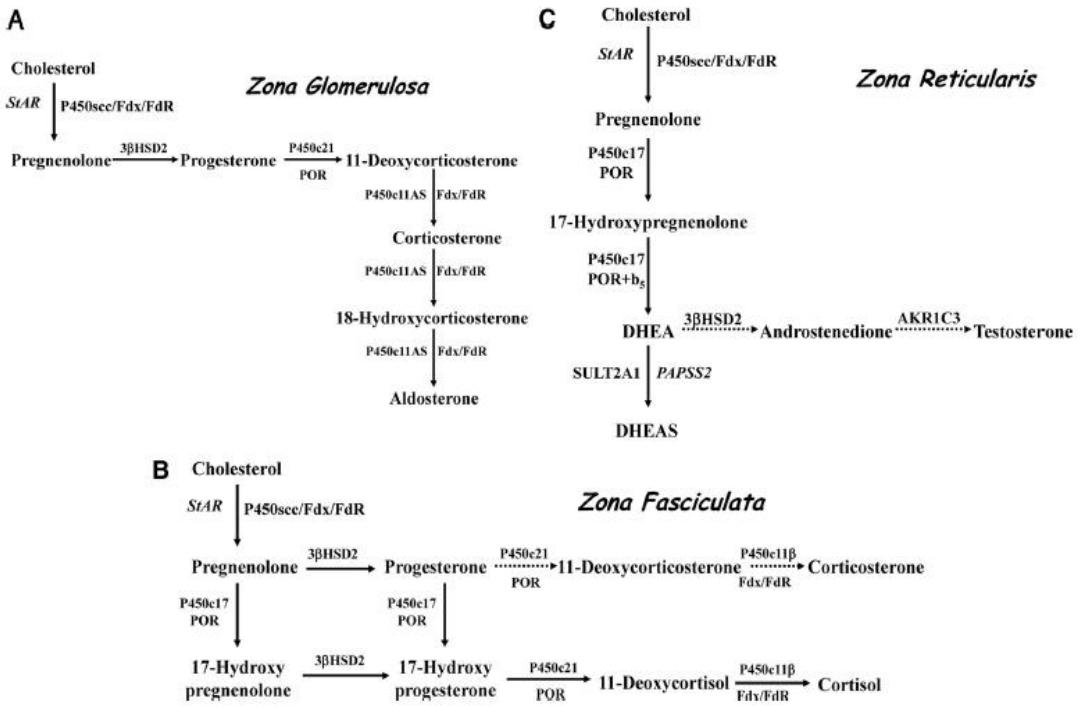


Figure 3.2. Steroidogenic pathways of the human adrenal cortex zones. Cholesterol is the initial substrate in all the zones. A) Biosynthesis of mineralocorticoids, mainly aldosterone, in the zona glomerulosa. B) Biosynthesis of cortisol and corticosterone in the zona fasciculata. Corticosterone is a minor glucocorticoid, synthesised in the absence of CYP17A1. C) Biosynthesis of androgen intermediates (dehydoepiandrosterone and dehydoepiandrosterone-sulfate) and androgen (testosterone) in the zona reticularis (Miller & Auchus 2011).

CYP11B1 is expressed in the steroidogenic pathway in the zona fasciculata (Figure 3.2B) and this enzyme is responsible for the final steps in cortisol production. CYP11B1 can not synthesise ALDO from its precursor 18-hydroxycorticosterone (18OH-CORT) because of on the zone-specific CYP11B1 and CYP11B2 isozyme expression. The CYP11B2 transcript is localised in the zona glomerulosa whilst CYP11B1 is expressed in the zona fasciculata/ reticularis (Rodriguez et al. 1997; Bassett et al. 2002). Cortisol production is ACTH regulated as the zona fasciculata expresses receptors for ACTH. The zona reticularis (Figure 3.2C) produces small amounts of cortisol as it expresses CYP21A2 and 11β-hydroxylase to a lesser extent than CYP17A1 which ensures the synthesis of dehydroepiandrosterone (DHEA) and dehydroepiandrosterone- sulfate (DHEAS) (androgen intermediates) (Auchus & Rainey 2004).

3.3. Adrenal steroid metabolism

The enzymes that facilitate synthesis of steroids are expressed in the gonads and adrenal glands. Two groups of steroidogenic enzymes catalyse steroid metabolism in the adrenal glands; these are cytochrome P450-dependent enzymes, (discussed in section 3.5), and hydroxysteroid dehydrogenases (HSDs). HSDs do not contain heme and require cofactors (NADH, NAD^+) to either reduce or oxidise steroids during steroid biosynthesis (Agarwal & Auchus 2005). Two HSDs, 3β-hydroxysteroid dehydrogenase (3βHSD) and 17β-hydroxysteroid dehydrogenase (17βHSD), take part in adrenal steroid biosynthesis (see Figure 3.3) (Payne & Hales 2004).

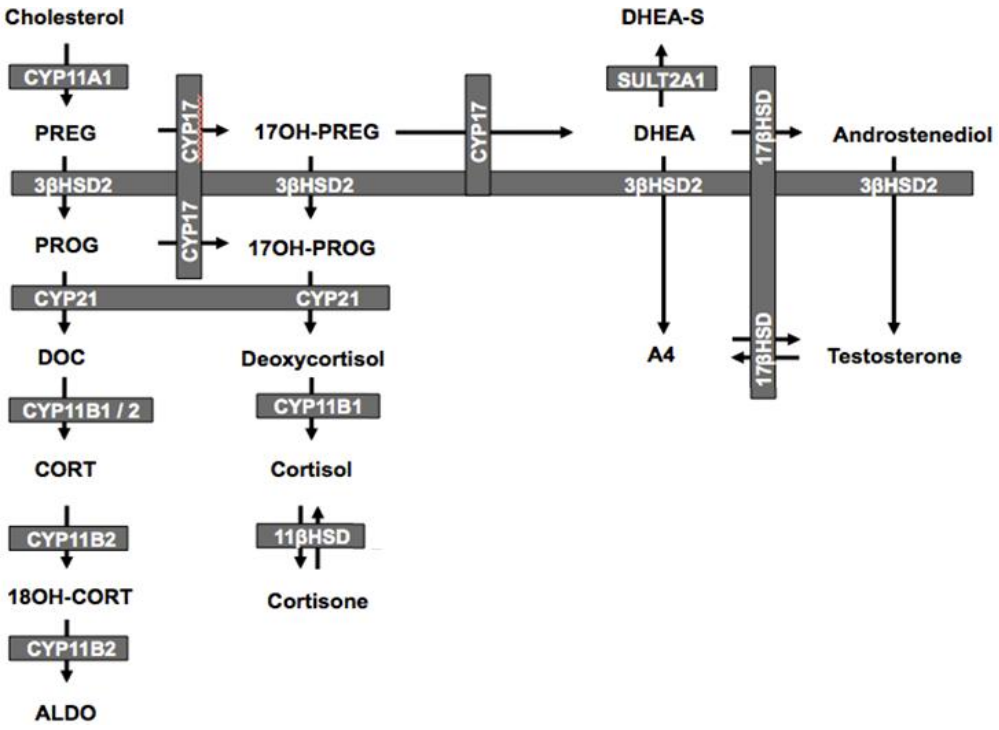


Figure 3.3. Schematic representation of enzymes and steroids in the steroidogenic pathway. PREG, pregnenolone (PROG), progesterone (DOC), deoxycorticosterone (CORT), corticosterone. 18 hydroxycorticosterone (18OH-CORT), aldosterone (ALDO), 17-hydroxypregnenolone (17-OHPREG), 17-hydroxyprogesterone (17-OHPROG), dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA).

The first step in adrenal steroidogenesis involves the cleavage of cholesterol by CYP11A1 enzyme located in the mitochondria, Figure 3.3. The rate limiting step of steroidogenesis is the uptake of cholesterol across the mitochondria membrane into the inner mitochondria membrane (Black et al. 1994). There three known sources of cholesterol which are, the *denovo* synthesis of acetyl CoA (acetyl coenzyme A), circulating low density lipoproteins

and hydrolysis of cholesteryl esters. Principally, cholesterol is obtained from circulating low density lipoproteins (Liu et al. 2000; Azhar & Reaven 2002). Cholesterol is hydrophobic and its uptake into the cells is mediated by the low density lipoproteins receptor which internalises the LDL via receptor mediated endocytosis. Once internalised, the low density lipoproteins is hydrolysed to free cholesterol by enzymes in lysozyme vesicles. The free cholesterol is transported to the mitochondria through the steroidogenic acute regulatory protein (StAR) transporter protein for steroid biosynthesis. Excess cholesterol is esterified and stored as acetyl CoA (Jeon & Blacklow 2006; Goldstein & Brown 2009). In rodents, the selective pathway (Figure 3.4) is used for high density lipoprotein instead of the endocytic pathway that is used in humans for low density lipoprotein (Azhar et al. 2003). ACTH, one of the tropic hormones, promotes acetyl CoA uptake in adrenal cells by stimulating a series of reactions as shown in Figure 3.4. The resulting steroids subsequently diffuse into the blood stream to be transported to their respective target tissues to elicit their effects (Mason & Rainey 1987; Brown et al. 1979; Gallegos et al. 2001).

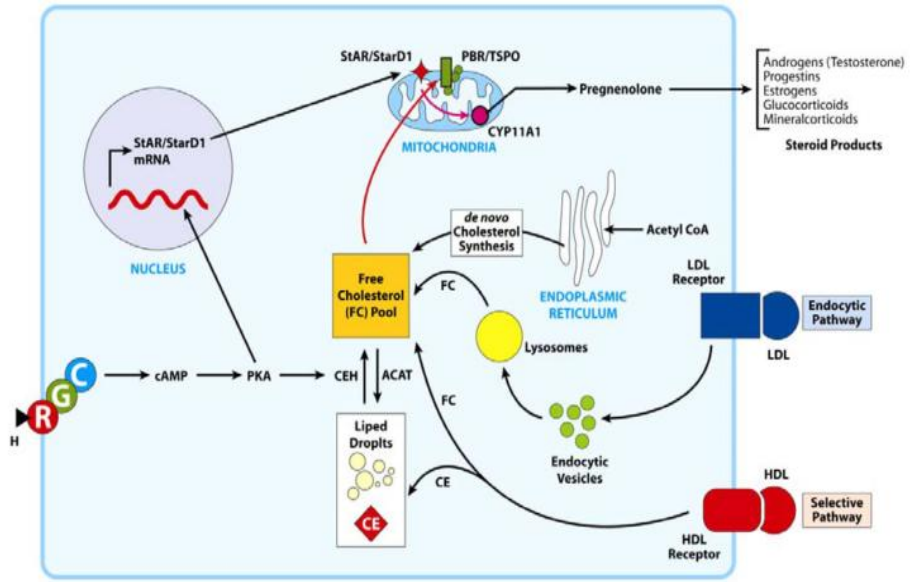


Figure 3.4. Cholesterol uptake and its metabolism for the biosynthesis of steroids in adrenal cortex cells. Free cholesterol (FC), acetyl CoA (CE), cyclic adenosine monophosphate (cAMP), high density lipoprotein (HDL), ACTH (H), low density lipoproteins (LDL), protein kinase (PKA), peripheral type benzodiazepine (PBR), cholesterol acyltransferase (ACAT), cholesterol ester hydroxylase (CEH), (Zaidi et al. 2012).

The initial substrate for steroidogenesis, cholesterol, is converted to PREG in the mitochondria as mentioned earlier in section 3.2 (see Figure 3.3). PREG diffuses into the endoplasmic reticulum (ER) to be substrate for either 3βHSD or CYP17A1. CYP17A1 has

two activities, a lyase and a hydroxylase activity. CYP17A1 subsequently converts PREG to 17α-hydroxypregnenolone (17OH-PREG) (hydroxylase) which is then converted to DHEA through the lyase activity. PREG, 17OH-PREG and DHEA then serve as substrates for 3βHSD, which converts these substrates to PROG, 17-hydroxyprogesterone (17OH-PROG) and androstenedione (A4) respectively, through the isomerase and dehydrogenase catalytic actions of 3βHSD. PROG then serves as substrate for CYP17A1 and CYP21A2. CYP17A1 catalyses the hydroxylation of PROG to 17OH-PROG and A4 while CYP21A2 converts PROG and 17OH-PROG to DOC and deoxycortisol respectively (Payne & Hales 2004). DOC and deoxycortisol diffuse into the mitochondria to be further metabolised to CORT, by CYP11B1 and CYP11B2, and to cortisol, by CYP11B1. CYP11B2 subsequently converts CORT to 18OH-CORT and then to ALDO, the principal mineralocorticoid (Mason & Rainey 1987; Swart et al. 1993; Albertin et al. 2002; Xing et al. 2011)

3.4. The properties of Cytochrome P450-dependent enzymes

Cytochromes P450 are heme containing membrane-bound proteins, associated with mitochondrial and endoplasmic reticulum (ER) membranes. They are found in all eukaryotes. The name of this group of enzymes is derived from the absorbtion peak observed at 450 nm when the reduced form of the enzyme is exposed to carbon monoxide (CO) *in vitro* (Omura & Sato 1964; Payne & Hales 2004).

The heme is penta-coordinated, with the tretradentate poryphyrin ring contributing the four ligands, see Figure 3.5. In the enzyme's substrate free state (ferric state), a cysteine residue from the polypeptide contributes the fifth ligand, which is a thiolate sulphur atom, while the sixth position is thought to be occupied by water. Upon reduction the sixth position becomes the site for dioxygen binding. For the steroidogenic cytochromes P450, the enzymes are named according to the oxygenation position on the steroid ring, for example CYP11 (11-hydroxylation), CYP17A1 (17-hydroxylation) and CYP21A2 (C21-hydroxylation) (Omura & Sato 1964).

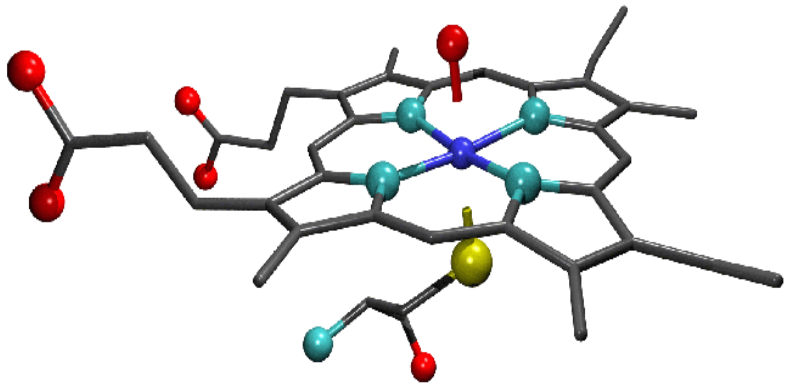


Figure 3.5. Active site of P450cam (CYP101) with no substrate bound. The light blue color represents nitrogen, while the red color represents oxygen. The iron is dark blue color and the sulphur is yellow. The carbon skeleton is in coloured grey (www.tcm.phy.cam.ac.uk).

The general catalytic cycle of P450 enzymes has been established and described in 9 steps (see Figure 3.6). The substrate binds to the iron in the active site inducing structural changes and a stable complex can be formed in the absence of reducing substances *in vitro*. During substrate binding, the iron will be in its ferric state (low spin). Upon substrate binding the distally coordinated water dissociates (step 1). The degree of substrate binding to the P450 active site can be determined using difference spectroscopy to record the changes in the spin state of the heme iron (De Montellano 1995). The heme iron changes from high spin state (Fe^{II}) to low spin state (Fe^{III}) due to the conformational changes to yield a type I spectrum (A), with an absorption maximum at 390 nm and an absorption minimum at 420 nm. Certain inhibitors of steroidogenic cytochromes P450 can bind to the active site of the enzyme and induce conformational changes to produce type II difference spectra with an absorption maximum at 430 nm and an absorption minimum at 390 nm.

The conformational changes caused by the substrate binding to the active site also result in the reduction of the redox potential of the heme iron. Conformational changes lead to the reduction of the ferric, Fe^{III}, to the ferrous, Fe^{II}, by a single electron derived from NADH via a specialised electron transfer chain (step 2). The reduction of Fe^{III} to Fe^{II} allows oxygen (dioxygen) to bind to the enzyme to form a ferric superoxide complex (step 3). Between steps 3 and 4 (step 3a), CO can interrupt the catalytic cycle by binding to the reduced P450 iron, yielding a type II or CO-induced difference spectrum. This spectrum has a peak maximum at 450 nm and can be used to determine the P450 concentration in suspensions or solutions (Omura & Sato 1964). Type I and type II spectra are discussed further in Chapter 4.

The introduction of a second electron (step 4) leads to the reduction of the ferric superoxide complex. Ferric superoxide complex is highly reactive and is protonated (step 5) to form a P450-peroxide complex. Addition of a second proton leads to the splitting of the dioxygen bond (step 6) with the release of one oxygen atom as a water molecule. The remaining oxygen atom forms an activated intermediate bound in the active site, which is inserted into the substrate as a hydroxyl molecule to form a hydroxylated product which is released from the active site (steps 7, 8 and 9). After hydroxylation, the enzyme returns to its original ferric form and the cycle is repeated (White & Coon 1980; Schliting et al. 2000). The intermediate formed at step 7 is believed to be the oxidative compound that was observed through a shunt pathway that uses hydrogen peroxide and benzoic acid as oxygen donors (Denisov et al. 2005). This pathway confirmed that the intermediate collects a hydrogen atom from the substrate to form a iron-hydrogen complex that recombines with the substrate to produce a hydroxylated compound that will form the product (White & Coon 1980; Denisov et al. 2005). Upon specific substrate or ligand binding to the cytochrome P450-dependent enzyme active site, P450 protein conformational changes associated with low spin and high spin states have been detected spectrophotometrically (Pcam et al. 1987; Wilker et al. 1999; Lewis 2005).

Cytochrome P450-dependent enzyme catalytic activity changes are influenced by a number of factors which include competition for electrons from electron donors in the presence of another P450 enzyme, ionic interactions and conformational changes of cytochrome P450-dependent enzyme structure (Ener et al. 2010; Sushko et al. 2012).

3.5. Electron transfer proteins of P450 enzymes

Cytochrome P450-depedent enzymes are also called external monooxygenases because they use two electrons transferred by an external donor (NADPH) for catalysis. Electron transfer from a protein donor to P450 is important to control the activity of the enzyme (Hintz & Peterson 1981; Brewer & Peterson 1988). As shown earlier in section 3.3, cytochrome P450-dependent enzymes catalyse hydroxylation reactions that involve substrate (RH) oxidation (addition of one oxygen atom) and reduction of the other oxygen atom to water (see Eq 1) (Munro & Lindsay 1996; Bernhardt 2006).

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+ \qquad \text{(Eq 1)}$$

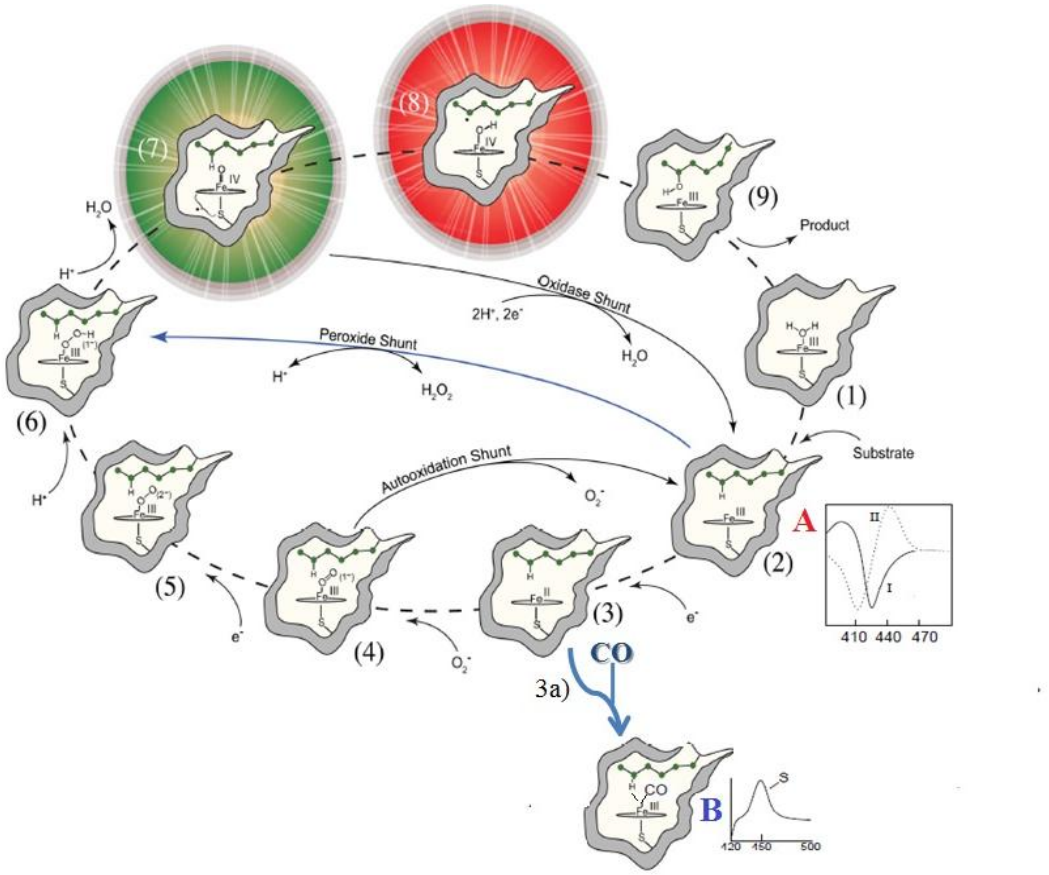


Figure 3.6. Catalytic cycle of cytochrome P450 enzyme. Reproduced and adapted from (Krest et al. 2013).

Adrenal cytochrome P450-dependent enzymes are also classified into groups according to their electron transfer chains. The microsomal steroidogennic cytochrome P450 enzymes, CYP17A1 and CYP21A2, are membrane-bound enzymes in the ER while the mitochondrial cytochromes P450, involved in steroidogenesis, are CYP11A1, CYP11B1 and CYP11B2 (Smith et al. 1994; Miller 2005). A schematic adrenal steroidogenesis diagram (see Figure 3.7), illustrates the adrenal steroidogenic pathway in mitochondria and the ER.

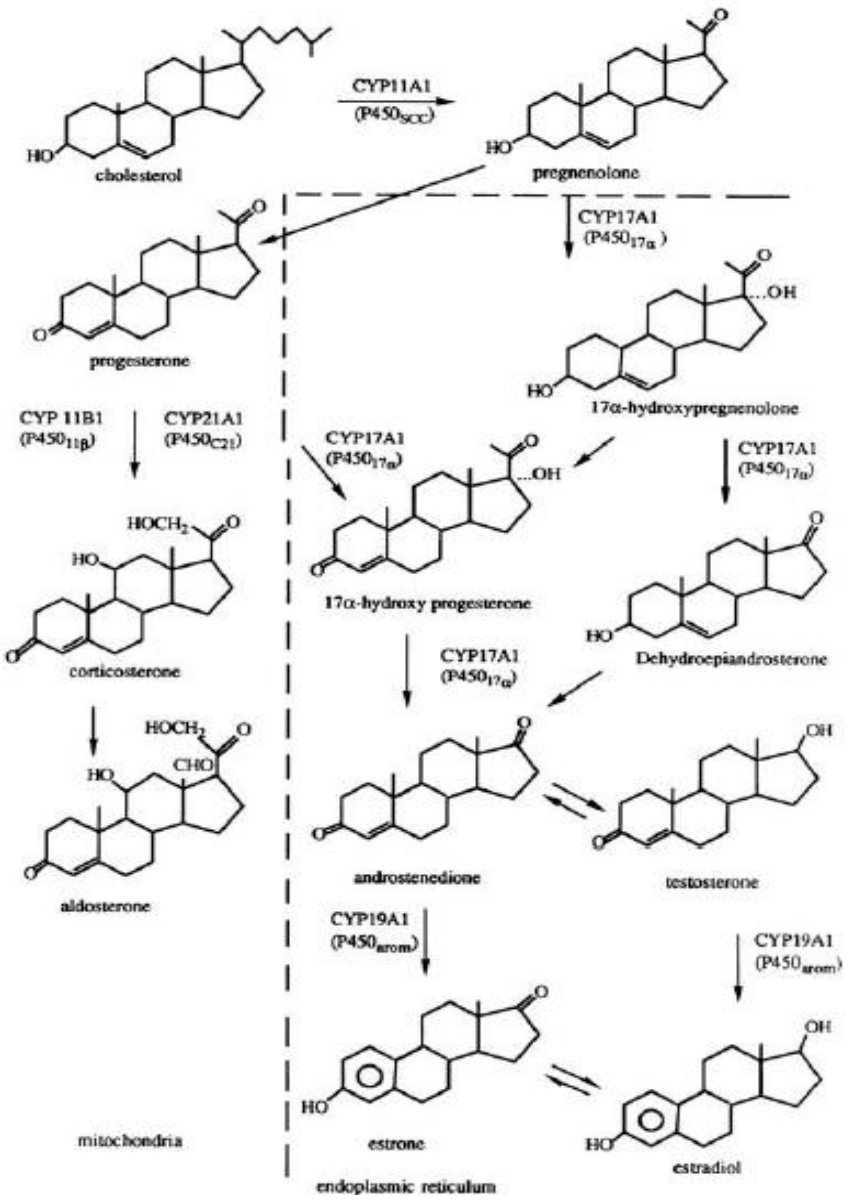


Figure 3.7. Steroid biosynthetic pathways in the adrenal cortex mitochondria and ER (Lewis 2005).

3.5.1. Microsomal cytochrome P450-dependent enzymes

Microsomal cytochrome P450-dependent enzymes are termed class II enzymes. These cytochrome P450-dependent enzymes receive their electrons from NADPH via cytochrome P450-oxidoreductase (POR). POR consists of a single polypeptide chain (Mr 78 000) and the active enzyme binds one mole of flavin mononucleotide (FMN) and one mole of flavin adenine dinucleotide (FAD) (Lu et al. 1969). During catalysis (see Figure 3.8), the FAD accepts a pair of electrons from NADPH to be reduced. The reduced FADH then transfers the electrons to FMN which subsequently releases the electrons, one by one, to the cytochrome

P450 during the catalytic cycle as previously described in section 3.5. This ensures that the reductase is a self-sufficient electron transfer unit, unlike the electron transfer system in type 1 enzymes that depend on another protein (Oparian & Coon 1982). As shown in Figure 3.8, cytochrome b_5, an electron transfer heme-protein, has been reported to have a differential stimulatory effect on cytochrome P450-dependent enzyme. Cytochrome b_5 can donate the second electron, but not the first one, to microsomal cytochromes P450 during enzyme catalysis (Auchus et al. 1998). The availability of electrons is a rate limiting factor in microsomal cytochrome P450-dependent enzyme reactions. The electron transfer protein is also a key component in drug metabolism by microsomal cytochrome P450-dependent enzymes in the liver (Hintz & Peterson 1981; Brewer & Peterson 1988). Chapters 4 and 5 report on investigations that were carried out on microsomal P450 enzymes, CYP17A1 and CYP21A2.

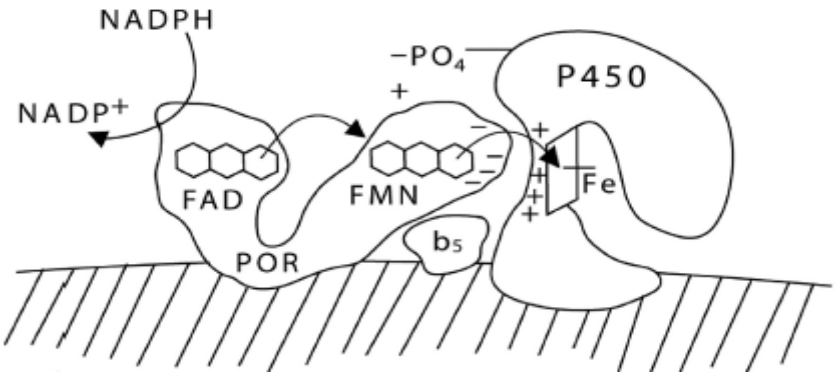


Figure 3.8. Microsomal protein electron transfer system. POR, cytochrome P450-oxidoreductase and b_5, cytochrome b_5. (Miller & Auchus 2011).

3.5.1.1. Cytochrome P450 17α-hydroxylase/17,20 lyase and cytochrome P450 21-hydroxylase

CYP17A1 and CYP21A2 are microsomal enzymes expressed in the gonads and adrenal glands and are responsible for the biosynthesis of androgens and mineralocorticoid and glucocorticoid precursors.

CYP17A1 is not expressed in the zona glomerulosa where ALDO, a mineralocorticoid, is synthesised, thus it can be assumed that this enzyme can determine adrenal zone specific steroidogenesis (Sparkes et al. 1991). It catalyses two reactions, the hydroxylation of PROG

to 17OH-PROG and PREG to 17OH-PREG as well as C17,20-lyase, or cleavage of the intermediate 17OH-PREG to DHEA.

The hydroxylation reaction utilises two electrons to reduce one atom of oxygen to water and oxidise the substrate (Bernhardt 2006). The CYP17A1 hydroxylase activity is the first activity, and promotes glucocorticoid formation, while the C17,20-lyase activity is the second activity that promotes androgen formation (Baston & Leroux 2007). Both the 17 α-hydroxylation and C17,20-lyase reactions occur at the same active site of CYP17A1 (Auchus et al. 1999). CYP17A1 and CYP21A2 are co-expressed in the zona fasciculata and their interactions are a mechanism for corticosteroid biosynthesis regulation, as they have the ability to inhibit each other, depending on their activities (Sushko et al. 2012). Cytochrome b_5 is a ubiquitous electron transport heme-protein that regulates the activity of CYP17A1. Cytochrome b_5 stimulates the C17,20 lyase activity, but not the hydroxylase activity, of CYP17A1 in the zona reticularis. It has been reported that in the absence of cytochrome b_5, CYP17A1 hydroxylase activity dominates, thus catalysing the production of negligible levels of sex steroids (Miller et al. 1997; Auchus et al. 1998).

The mechanism of action of CYP17A1 is not yet fully understood, thus CYP17A1 has become a target for investigations. CYP17A1 is one of the P450 enzymes that undergoes phosphorylation and post-translational modification, which influences its rate of activity. CYP17A1 undergoes phosphorylation, and this mechanism has been hypothesised to be associated with some clinical conditions such as hyperandrogenism and insulin resistance (Zhang et al. 1995; Pandey et al. 2003). Defects of both CYP17A1 hydroxylation and C17,20-lyase reactions, due to mutations, lead to decreased levels of sex and glucocorticoid steroids, and mineralocorticoid excess. The decrease in sex steroids and glucocorticoids leads to sexual development disorders and hypertension (Hwang et al. 2011).

CYP21A2 catalyses the hydroxylation of PROG and 17OH-PROG to DOC and deoxycortisol, which is the key step in glucocorticoid and mineralocorticoid formation. It is only encoded for by one gene and uses the same electron transport protein system that CYP17A1 uses (Scott et al. 2007). Unlike CYP17A1, CYP21A2 is expressed in all adrenal zones but only influences the production of corticosteroids (Payne & Hales 2004). CYP21A2 deficiency leads to low blood pressure due to decreased cortisol and ALDO levels. Low

blood pressure has devastating clinical effects leading to conditions that include fatal cardiovascular diseases (Clayton 2002).

3.5.2. Mitochondrial P450 enzymes

Mitochondrial P450 enzymes are considered to be type I enzymes and they include CYP11A1, CYP11B1, and CYP11B2. These enzymes catalyse hydroxylation reactions in steroid biosynthesis. Unlike the type II P450 enzymes, type I enzymes depend on two electron transfer proteins, adrenodoxin (ADX) and adrenodoxin reductase (ADXR) denoted as Fdx and FdR respectively, see Figure 3.9. ADX and ADXR form a linked biomolecular complex. ADXR is an insoluble iron-sulphur protein, embedded in the inner membrane of the mitochondria together with the P450 enzyme. ADXR is a flavin moiety which accepts electrons (reduction of the ADXR) from NADPH and delivers them to ADX, which then dissociates from ADXR to deliver the electrons to the P450 enzyme. ADX thus functions as an electron shuttle between the ADXR and the P450 enzyme (Kimura & Suzuki 1967; Ziegler et al. 1999).

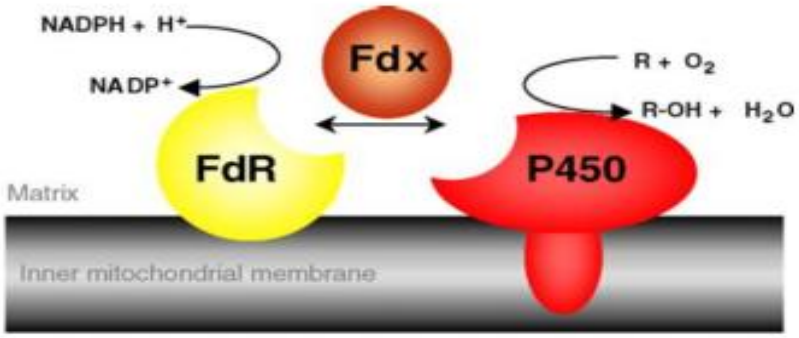


Figure 3.9. Mitochondria protein electron transfer system. Adrenodoxin (Fdx), adrenodoxin reductase (FdR), (Bernhardt 2006).

3.5.2.1. Cytochrome 11β-hydroxylase and aldosterone synthase

CYP11B1 and CYP11B2 enzymes, also referred to as P450c11 enzymes, are located in the mitochondrial membrane of all three adrenal zones. The mitochondrial enzymes metabolise the final steps of glucocorticoid and mineralocorticoid biosynthesis (Fardella & Miller 1996). CYP11B1 catalyses the hydroxylation of deoxycortisol to cortisol while CYP11B2 catalyses a three step reaction that produces CORT, 18OH-CORT and ALDO (Imai et al. 1998). CYP11B1 is the most abundant P450c11 enzyme, expressed predominantly in the zona

fasciculata and to a lesser extent in the zona reticularis (Nishimoto et al. 2010). CYP11B2 is able to convert DOC to ALDO because it is only expressed in the zona glomerulosa where it has 11β-hydroxylase, 18-hydroxylase and 18-methyloxidase activities (Ogishima et al. 1991; Curnow et al. 1991). It has been noted that CYP11B1 and CYP11B2 can convert DOC to CORT and 18OH-CORT, respectively, but CYP11B1 cannot convert 18OH-CORT to ALDO (Mulatero et al. 1998). Studies on P450c11 have shown that both enzymes prefer to bind to DOC than intermediates (CORT and 18OH-CORT) and 18OH-CORT are not released from their active sites during steroid synthesis (Imai et al. 1998). Mutations of P450c11 can lead to congenital adrenal hyperplasia, which is overproduction of cortisol that leads to hypertension and hyperaldosteronism (Zachmann et al. 1983).

3.6. Steroids and their receptors

Steroids are important biological compounds which mediate a wide range of physiological processes such as cellular differentiation and proliferation, control of metabolism and immunity, development of sexual characteristics, muscle and bone synthesis, maintenance of water and salt concentration as well as control of reproductive system. There are five classes of steroids recognised in mammals: mineralocorticoids, glucocorticoids, androgens, estrogens and progestagens. In this section, only mineralocorticoids, glucocorticoids, and androgens are discussed. The mineralocorticoids are important as they maintain water and mineral balance. The glucocorticoids regulate the metabolism of carbohydrates, lipids, and proteins while the androgens play a vital role in sexual development and growth.

The adrenal cortex zones are the primary steroidogenic sites where mineralocorticoids, glucocorticoids, and androgens are biosynthesised. Adrenal steroids are all biosynthesised from cholesterol via sequential cytochrome P450 steroid hydroxylases and 3βHSD catalysed reactions (known as steroidogenesis) (Li et al. 2004). Steroids are lipid soluble and thus pass through the lipid membranes by diffusion out of and into the blood stream. In the cytoplasm, steroids bind to their respective receptors in the cytoplasm or nucleus to form steroid-receptor complexes (White 1994).

Glucocorticoid receptors and androgen receptors not in a steroid-receptor complex, are mostly found in the cytoplasm, and translocate to the nucleus only after forming a steroid-receptor complex. The mineralocorticoid receptor is found in both the cytoplasm and nucleus

when not in a steroid-receptor complex, but only found in the nucleus when complexed to a steroid (Tyagi et al. 2000). Steroid receptors travel to and from cellular compartments as they mature to exert their biological effects. The receptors are associated with chaperone and co-chaperone proteins in the absence of steroids. Receptors act as ligand-dependent transcription factors that regulate various genes in the presence of their respective steroids (Echeverria & Picard 2010). Chaperones are important for the maintenance of the receptors in an active and steroid responsive form, and dissociate from the receptor when a specific steroid binds (Pratt et al. 2004; Echeverria & Picard 2010). Chaperones and co-chaperones (Hsp90 and Hsp70 respectively) are proteins that prevent protein aggregation of steroid receptors (Zuehlke & Johnson 2011).

Some steroids (see L in Figure 3.10) elicit their effects when they diffuse into the cytoplasm, where they bind to a specific steroid receptor (glucocorticoid, mineralocorticoid, or androgen receptor) that is associated with the chaperone. Upon binding, the steroid-receptor complex dissociates from the chaperones, undergoes conformational change, and moves to the nucleus. The receptor part of the steroid-receptor complex binds to the steroid response element on the DNA which induces target gene activation by transactivation or trans-repression, see Figure 3.10. The mineralocorticoid steroid mechanism of eliciting its effect is similar to the glucocorticoid steroid mechanism (Pratt et al. 2004) .

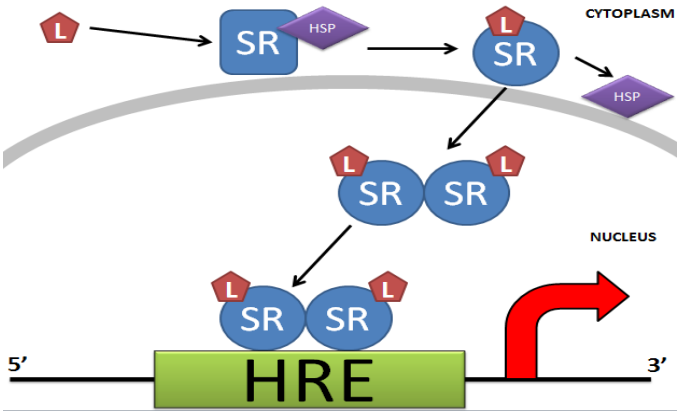


Figure 3.10 Representation of gene activation via the steroid receptor (SR) upon ligand (L) binding. Heat shock proteins (HSP), hormone response element (HRE).

3.6.1. Mineralocorticoids

Mineralocorticoids are produced in the zona glomerulosa (Xing et al. 2011). A number of factors regulate the biosynthesis of the primary mineralocorticoid, ALDO. Principally

production of ALDO is regulated by Ang II, ATCH and a decrease in sodium levels and an increase in potassium levels in the blood (Pascual-Le Tallec & Lombès 2005). The other active mineralocorticoids are DOC, CORT and 18OH-CORT, but are considered weak mineralocorticoids because their known potencies are lower than ALDO (Kazuhiko et al. 1987). Mineralocorticoids regulate fluid and salt balance by inducing reabsorption of sodium ions and excretion of potassium ions, mostly in the kidneys and colon. ALDO is responsible for maintaining acid-base balance by inducing reabsorbtion of the H^+ ions in the collecting duct, resulting in urine acidification (Good 2007). The mineralocorticoids have been shown to stimulate synthesis of cardiac collagen and proliferation of fibroblast in the heart (Padmanabhan et al. 2000).

Cortisol, a glucocorticoid, has been reported to also bind and activate the mineralocorticoid receptor. Though the mineralocorticoid and glucocorticoid receptors display close structural and functional similarities, ALDO is selective for the mineralocorticoid receptor only (Pascual-Le Tallec & Lombès 2005). Cortisol and ALDO bind to the mineralocorticoid receptor with similar affinities but their dissociation kinetics differ, where that of ALDO is slower showing that ALDO-receptor complexes are more stable than cortisol-receptor complexes (Lombes et al. 1994). The dehydrogenase enzyme, 11βHSD type 2, protects the mineralocorticoid receptor from cortisol binding. This enzyme catalyses the conversion of cortisol and CORT to their inactive metabolites, cortisone and 11-dehydrocorticosterone (11-DHC), in humans and rodents respectively. The enzyme is expressed in the colon and kidney which are mineralocorticoid target tissues. Impaired activity of this enzyme results in sodium retention (Stewart et al. 1996).

Clinical conditions, such as hypertension, have been linked with high concentrations of ALDO. CYP11B2 deficiency, due to mutations, lead to salt wasting and hypotension (Padmanabhan et al. 2000). Competitive inhibitors of angiotensin-converting enzyme (ACE), such as enalapril, and mineralocorticoid receptor inhibitors, such as spironolactone, have been used for the treatment of hypertension. Inhibitors that directly inhibit some of the P450 enzymes that synthesise ALDO are still being investigated (Abrams et al. 1984; Sturrock et al. 2004; Calhoun et al. 2011).

3.6.2. Glucocorticoids

Biosynthesis of glucocorticoids occurs in the zona fasciculata of the adrenal cortex. Glucocorticoids are considered to be stress hormones since their secretion is altered stress factors (Huizenga et al. 1998; Chrousos & Gold 1998). Secretion of glucocorticoids is controlled by the HPA axis in response to various physiological and physical stress factors. Secretion from the adrenals occurs in a pulsatile ultradian rhythm. The main glucocorticoid is cortisol in humans and CORT in rodents. Glucocorticoids derive their name from one of their functional roles in the body, which is the stimulation of glucose metabolism (Talabér et al. 2013).

Glucocorticoids are steroids that control transcription of many genes that affect a number of important metabolic and inflammatory reactions. They are responsible for increasing the blood glucose levels by coordinately controlling the expression of genes involved in amino acid degradation, ammonia detoxification and gluconeogenesis. Glucocorticoids induce the expression of anti-inflammatory proteins (cytokines) and suppress expression of pro-inflammatory proteins (Rhen & Cidlowski 2005; Permissive & Munck 2000). The importance of glucocorticoids is reflected in the inability of humans to survive without them. Abnormal glucocorticoid production, such as a cortisol deficiency due to adrenal insufficiency, leads to weight loss, tiredness, weakness and impaired regulation of blood glucose levels (Stewart et al. 1993).

Chronic stress results in elevated cortisol levels which have been associated with clinical conditions such as hypertension, metabolic syndrome cardiovascular diseases, hyperglycemia, suppressed immunological responses and monocyte differentiation into macrophages (Elenkov & Chrousos 2002). Patients suffering from connective tissue disorders, bullous diseases, and other dermatological conditions, are treated with systematic long-term courses of glucocorticoids. However, it has been shown that long-term glucocorticoid therapy causes adverse results in bone turnover, lung differentiation, cell growth, and proliferation during wound healing. Also glucocorticoid therapy affects the HPA axis as it reduces the hormone levels of sex steroids (Curtiss Jr et al. 1954). Therefore maintaining normal glucocorticoid levels is critical to the endocrine function.

3.6.3. Androgens

Androgens, C19 steroids, are produced in the zona reticularis of the adrenal cortex. DHEA is a pre-androgen that is secreted in the ovaries and adrenals in women before menopause. The zona reticularis expresses large amounts of CYP17A1, which in turn results in large amounts of DHEA. The DHEA is subsequently converted to A4 and DHEAS through sulphonation. The DHEAS and A4 are precursors for the production of the sex steroids testosterone and estrogen in women. DHEA and DHEAS are high circulating androgens but are weak androgens as they have a lower affinity for the androgen receptor than testosterone. Testosterone, a potent androgen, is biosynthesised in small amounts (Handa & Price Jr 2000). Levels of sex steroids are controlled by the expression of other steroidogenic and metabolising enzymes which determine the conversion of DHEA to potent sex steroids in each tissue type. This allows for local steroid hormone activity as the steroids do not diffuse out of the tissues into the blood circulation, thus reducing side effects on other tissues (Panjari & Davis 2010).

The use of these adrenal androgens is not well understood. It is reported that their increase is linked to the development of secondary sex traits such as pubic hair and contribute to some of the body changes at an early age (Idkowiak et al. 2011). DHEA deficiency is usually a result of clinical conditions such as Addison's disease which inhibit adrenal steroid synthesis which is treated by DHEA replacement therapy (Buvat 2003).

3.7. Regulation of steroidogenesis

Regulation of steroidogenesis depends on hormones produced primarily by the neuro-endocrine system. Regulation of steroid biosynthesis is important as the steroids are not stored in the body but rather produced when needed (Dickerman et al. 1984). To maintain the complex hormonal systems, there are complex mechanisms which regulate hormone release to maintain homeostasis. A series of positive and negative feedback activities exist for these systems. The HPA axis and the RAAS are well known regulatory mechanisms that incur negative and positive feedback when necessary. The regulation can be acute or chronic where acute response occurs within minutes from ACTH, Ang II and potassium ion stimulation. This stimulation results in the mobilisation of cholesterol for streoidogenesis. The circulating ACTH, Ang II and potassium ion concentrations influence the capacity of adrenal

steroidogenesis, where glucocorticoid, mineralocorticoid and androgen biosynthesis is regulated (Li et al. 2004). Chronic responses can take hours and involves transcription and expression of steroidogenic enzymes (Clark et al. 1995). Unregulated steroid biosynthesis can lead to adverse clinical conditions such as hypertension, kidney failure, heart diseases and many more.

ACTH and Ang II are the primary effectors of adrenal cortex steroidogenesis where ACTH plays a major role in the HPA axis and Ang II plays a major role in RAAS. Regulation of steroidogenesis occurs in different ways which include inhibition or stimulation of enzyme expression. The peptide hormone, ACTH, has been reported to have the ability to regulate P450c11 gene expression as it stimulates CYP11B1 transcription, a process that can take hours (Arola et al. 1994). Moreover, in the long term, ACTH inhibits gene expression of CYP11B2 and thus subsequently inhibits ALDO synthesis (Aguilera et al. 1996). Some studies have shown that ACTH eliminates intermediates for ALDO biosynthesis by increasing the expression of CYP17A1 and CY11B1 that produce cortisol. The zona glomerulosa maintains CYP11B2 gene expression under normal ATCH circulating levels. This is due to Ang II inhibiting ACTH's stimulating effects on the zona glomerulosa and not on the zona fasciculata (Begeot et al. 1988). Proliferation of zona fasciculata cells and transformation of zona glomerulosa to zona fasciculata cells has been reported to be induced by ACTH (Arola et al. 1988). The regulatory mechanisms (HPA axis and RAAS) are discussed in the following sections.

3.7.1. Hypothalamic–pituitary–adrenal axis

The HPA axis (Figure 3.11) is a complex regulatory system which integrates the endocrine system and the neuronal systems. The integration of the two systems makes the HPA system important as it affects the immune system and the central nervous system (Watson & Mackin 2006). The system consists of the hypothalamus, the pituitary and the adrenal gland. The HPA axis, as explained earlier in section 3.1, is controlled by secretion of CRH and AVP hormone from the hypothalamus, the primary gland. The secretion is initiated by internal or external physiological stimuli, which are detected by the nervous system. The physiological stimuli, which can be referred to as stressors, can be a drop in glucose plasma levels, or abnormal body temperature (Munch 1971).

CRH and AVP act together to stimulate production and secretion of ACTH by the anterior pituitary gland. The CRH has been reported to be only active in acute stress while AVP is important for the maintenance of the HPA axis during chronic stress (Lightman 1994; Bonaz & Rivest 2004). AVP cannot function on its own as it is a weak ACTH release stimulant but has been reported to aid CRH to stimulate ACTH secretion (Liu et al. 2004). AVP also has been reported to stimulate re-uptake of water by the kidneys as it increases the permeability of the collecting ducts (Vander et al. 2004).

The hypothalamus and the anterior pituitary gland are connected together by a vascular system. The vascular system ensures blood flow from the hypothalamus to the pituitary gland thus CRH and AVP are transported to the pituitary gland (Vander et al. 2004). The anterior pituitary gland secretes ACTH directly into the main blood stream to be transported to the adrenal cortex. At the adrenal cortex, ACTH stimulates steroidogenesis, particularly glucocorticoid synthesis, by binding to the ACTH receptors on the cell membranes of the adrenal cortex. The HPA axis help to maintain constant glucose levels in the body.

The HPA axis is self-controlled by unbound glucocorticoids in the blood stream to reduce fluctuations of glucocorticoids, this is referred to as the negative feedback mechanism (Figure 3.11). The unbound glucocorticoids act on the central nervous system, the hypothalamus and the pituitary gland thus inhibiting synthesis and secretion of CRH and ACTH (Meyer et al. 1998).

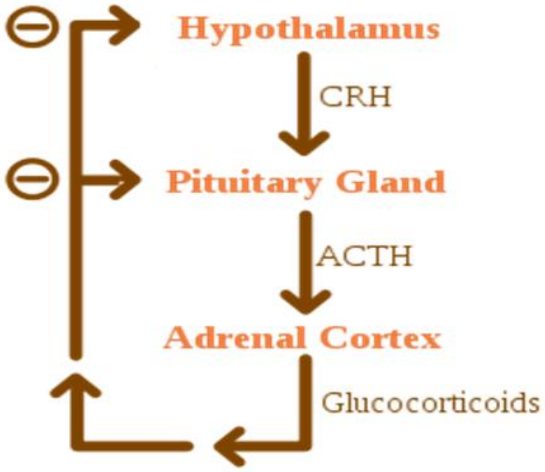


Figure 3.11. Representation of the hypothalamus-pituitary-adrenal axis and its negative feedback mechanism. Reproduced and adapted from (Mitrovic 2002)

3.7.2. Renin-angiotensin–aldosterone system

The RAAS (see Figure 3.12) is a system that is important for the regulation of steroidogenesis in the adrenal cortex, particularly ALDO biosynthesis. Maintaining blood pressure and intravascular fluid volume is the main function of the RAAS. The system consists of renin, an aspartyl proteolytic enzyme produced for its precursor, prorenin. Prorenin is cleaved to renin in the renal afferent arteriole juxtaglomelur cells of the kidneys. Renin secretion is stimulated by reduced perfusion blood pressure in the afferent arteriole which is sensed by baroreceptors. Biosynthesis of renin is inhibited by increased renal perfusion blood pressure and increased circulation of Ang II (Urata et al. 1990; Bock et al. 1992). High blood plasma levels of K^+ stimulate ALDO biosynthesis and secretion and suppress renin's activity. High salt concentrations in distal tubules of the kidney increase vasoconstriction of the afferent arteriole and decrease renin release, which consequently leads to decreased ALDO biosynthesis (Harrison-bernard 2009).

Low blood pressure could be a result of loss of water from the blood or low concentrations of salts namely sodium (Na^+) and potassium (K^+). Secretion of renin is the rate-limiting step of RAAS and responds to decrease in renal blood pressure and salt content. Renin cleaves hepatic angiotensinogen to Angiotensin I (Ang I) in the liver. Ang I (which is inactive) is primarily converted into Ang II in the lungs by ACE (Zaman et al. 2002; Wu et al. 2010). Ang I can be converted to Ang II by protease chymase, a chymotrypsin-like protease (Urata et al. 1990).

Ang II, a peptide, forms an important part of the system, as it stimulates the adrenal cortex to produce steroids, in particular mineralocorticoids. The peptide Ang II can be locally produced by various tissues in the body, including the heart, which could be responsible for the chronic maintenance of cardiovascular homeostasis (Engelia et al. 2003; McMurray et al. 2010). Ang II activates the Ang II type 1 receptor (AT_1) which causes vasoconstriction and raises blood pressure (Figure 3.12). Ang II binds to the AT_1 receptors on the adrenal cortex, preferably on the zona glomerulosa, to elicit ALDO production and secretion (Ehrhart-Bornstein et al. 1998; Briet & Schiffrin 2010). The main mineralocorticoid, ALDO, in turn, elicits a series of reactions via mineralocorticoid receptor activation to restore normal water and salt balance. ALDO increases renal reabsorption of water and Na^+ (via the epithelial

sodium channel) while stimulating the elimination of potassium in the kidney. Therefore ALDO helps to maintain blood pressure and volume to vital organs (Carpenter et al. 1961). Plasma concentrations of Ang II, Na^+ and K^+ are the key factors that stimulate RAAS activity (Ehrhart-Bornstein et al. 1998). Low blood plasma levels of Na^+, probably due to a low Na^+ diet, stimulate an increase in renin and ALDO plasma concentration (Takagi et al. 1988).

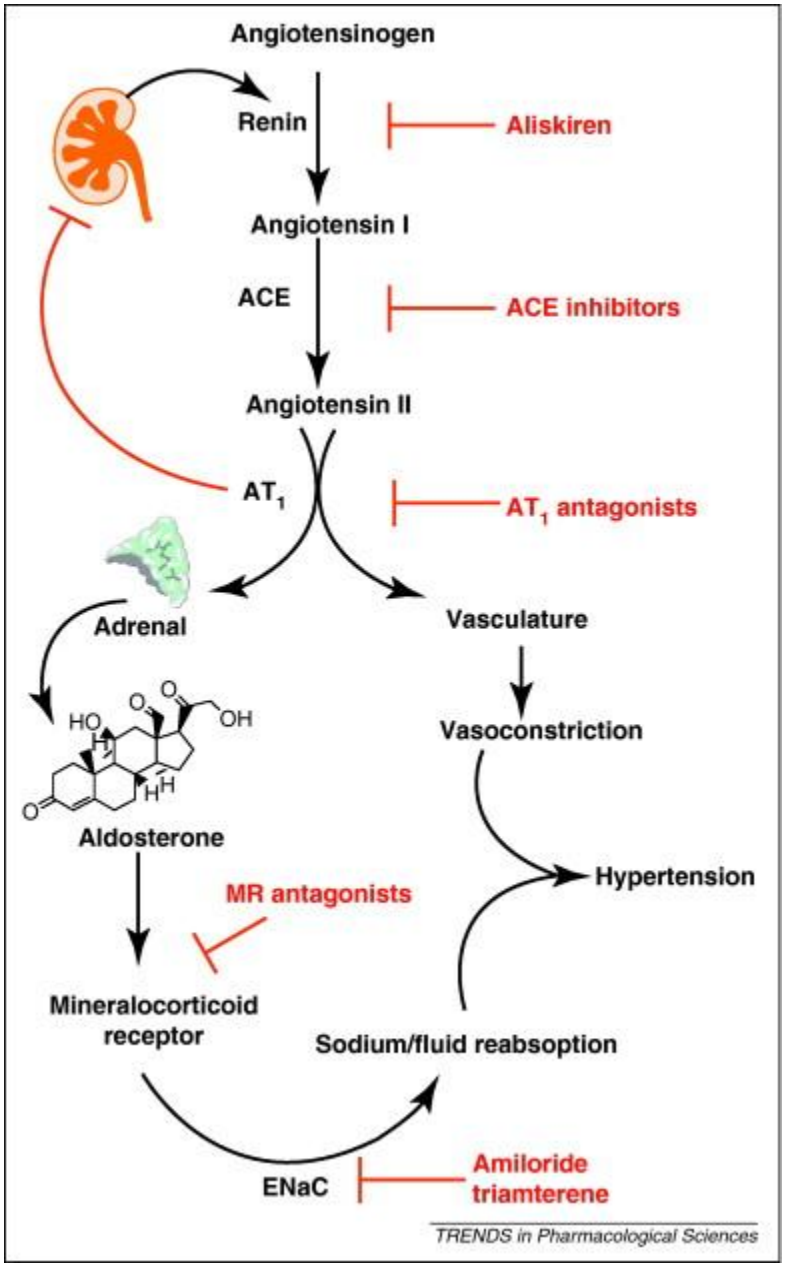


Figure 3.12. Schematic representation of RAAS and its inhibitors. (Sayer & Bhat 2014).

High blood pressure (hypertension), kidney diseases, and cardiovascular diseases have been found to be assorciated with the malfunction of RAAS. Malfunction of RAAS could be due

to overproduction or underproduction of renin or Ang II or ALDO which leads to clinical disorders. A study conducted by Bird et al. (1992) in ovine adrenal cells showed that Ang II stimulate production of ALDO and not cortisol by suppressing CYP17A1 expression and rendering the adrenocortical cells irresponsive to ACTH. This study showed that Ang II has the ability to inhibit glucocorticoid secretion (Bird et al. 1992). Results of another investigation carried out in human adrenocortical cells showed that Ang II stimulation, not only resulted in ALDO secretion but in cortisol secretion (Bird et al. 1992).

Currently inhibitors such as ACE inhibitors (lisinopril, captopril), renin inhibitors (aliskerin) and mineralocorticoid antagonist (spironolactone, eplerenone) are used to treat high blood pressure (see Figure 3.12). It has been reported that weight loss in humans decrease the circulating concentration of angiotensinogen, plasma renin, and ALDO (Engeli et al. 2005; Dall'Astaa et al. 2009). Therefore maintaining a healthy life style is vital for the prevention of hypertension. Usage of the ACE inhibitors mentioned earlier has side effects such as impotence, gynaecosmastia, breast tenderness, and menstrual irregularities. These drugs interfere with PROG and androgen receptors hence there is a need to develop a drug that has fewer side effects or none at all (Menard 2004).

Regulation of androgen synthesis is not well known, although the results of some studies indicate that the zona fasciculata and reticularis preferentially regulate expression of CYP17A1 and CYP11A1 together with 3βHSD and cytochrome b_5. These enzymes ultimately control and determine androgen production (Rainey & Nakamura 2008). ACTH also stimulates androgen production but there are additional unknown mechanisms responsible for the regulation of androgen production. ACTH adrenal stimulation has sensitive negative feedback control via the effect on CRH production by cortisol only and for androgens it is unknown what effect it has (McKenna et al. 1997).

3.8. Inhibition of P450 enzymes

P450 enzymes are important enzymes because of the number of metabolic functions they exhibit, as discussed in the earlier sections. These enzymes are involved in detoxification of chemical compounds and drugs that might otherwise cause adverse effects, such as cancer, allergies or intolerance reactions. Drugs interact with the enzymes either by being metabolised or inhibited. The role of P450s in bio activation, and metabolism of drugs

leading to prevention and treatment of ailments such as tumor formation, has made these enzymes targets for drug treatments (Baston & Leroux 2007).

Inhibitors are drugs that block the activity of one or more metabolic activities of enzymes and the degree of inhibition depends on the ability of the inhibitor to bind to the active site of the enzyme or the inhibitor's concentration. A single P450 enzyme can metabolise a particular drug and, simultaneously, be inhibited by that particular drug. On the other hand, a drug can be metabolised by one P450 enzyme and inhibit another enzyme. Inhibition is usually immediate unlike induction of enzyme activity that takes time (Lynch & Price 2007).

P450 inhibition is categorised as reversible, quasi reversible and irreversible. Reversible is the most common and documented type of drug interaction. Reversible inhibition only shows dose-dependent inhibition kinetically. Normal metabolic activities of the reversibly inhibited enzymes resume after elimination of the inhibitor in the body. Reversible inhibition is divided into competitive and non-competitive inhibition: where competitive inhibition entails the inhibitor preventing binding of the substrate to the active site while non-competitive binding is when the inhibitor does not affect substrate binding as it binds to another site that is not the active site (Lin & Lu 2001).

Quasireversible inhibition is when a stable, non-covalent and tight metabolic intermediate P450 complex is formed. The complex does not destroy the enzyme but the enzyme will be unavailable for any other metabolic activities. Unlike in *in vivo* conditions, the metabolic complex formed is considered irreversible in *in vitro* conditions. Irreversible inhibition is when the enzyme is inactivated or destroyed by the inhibitor due to the covalent binding of the reactive intermediate to the heme of the P450 active site. Irreversible inhibition only shows dose-dependent and time-dependent inhibition kinetically. In order to correct the quasireversible and the irreversible inhibition *in vivo*, new enzymes will have to be synthesized (Lin & Lu 2001).

Non-steroidal compounds, which are compounds that inhibit enzymes through reversible competitive inhibition, are now being used as P450 inhibitors as they do not cause unwanted side effects. Steroidal compounds have affinity towards one or more SRs acting as antagonists or agonists, thus they are not specific and result in adverse side-effects (Baston & Leroux 2007). The non-steroidal inhibitors have a sterically non-hindered heteroatom in their

structure which controls the heme and they have hydrophilic groups that mimic the natural substrate of enzymes. For instance, a non-sterodial inhibitor, Fadrozole, used to inhibit aromatase, is selective and more potent and influences both ALDO and CORT synthesis. Side-effects are avoided when the inhibitor is sufficiently specific not to induce unnecessary steroid biosynthesis of other steroids (Baston & Leroux 2007).

3.9. Conclusion

The adrenal gland is an important organ of the body that is part of the network that controls homeostasis. It is involved in steroidogenesis, which is a complex and vital process in mammals. Steroidogenesis yields steroids responsible for maintaining homeostasis and is regulated by various complex mechanisms. Cholesterol, the main substrate for steroidogenesis, is converted to various steroids. Cytochrome P450 enzymes together with dehydrogenases catalyse the production of steroidogenesis. The enzymes' catalytic activity determines the steroid output. Investigating the homology and activity of P450 enzymes has become important because these enzymes are not only involved in steroid biosynthesis but also drug metabolism. P450 enzymes' catalytic activity depends on the electron donors they are involved with and they have been categorised according to where their electron donors are localised (mitochondria and microsomal P450 enzymes). The mitochondrial and microsomal enzymes produce different steroids and they are dependent on each other. The RAAS and the HPA axis are important for the regulation of steroidogenesis as they control steroid production by controlling steroidogenic enzymes expression in the adrenal. Understanding the regulation of their expression, activity, and inhibition mechanisms is important for pharmaceutical reasons.

P450 enzymes, like any other enzymes, are specific to their endogenous substrates; therefore their inhibitors could be chemically or structurally similar to their endogenous substrates. The inhibitors will bind to the active site or to another pocket on the enzyme and cause a conformational change like the substrate would do. Available P450 enzyme inhibitors currently used to alleviate endocrine related diseases are competitive inhibitors and they inhibit P450 steroid biosynthesis activity. These drugs have been found not to be specific for the enzyme they are supposed to inhibit but interfere with the activity of other P450 enzymes and other enzymes triggering toxicological effects and unwanted side-effects. Drugs, such as

spironolactone, interfere with PROG and androgen binding to their respective receptors thus causing sexual undesirable effects (Menard 2004). These effects have led to the need to find drugs or compounds that can be used to inhibit the activity of P450 enzymes without causing unwanted side-effects.

To date, there has not been a drug that has been approved for human usage that can specifically inhibit CYP11B2 which produces ALDO. A drug, LC1699, has been reported to decrease ALDO tissue and plasma concentration in primary aldosteronism. The drug was tested on patients with primary aldosteronism and it was shown that at a concentration of 0.5-1 mg, there was a dose-dependent reversible suppression of plasma and urinary ALDO concentration by 70-80 %. In the same study it was also noted that there was a dose-dependent increase of Ang II and deoxycortisol concentration; thus this drug was not approved for human use as it inhibited other enzymes' activities, such as that of CYP11B1 (Amar et al. 2010; Calhoun et al. 2011).

Chapters 4 and 5 report the effect of Rooibos extract on microsomal and mitochondrial P450 enzymes particularly CYP17A1, CYP21A2, CYP11B1, and CYP11B2. Chapter 5 also reports on investigations into the influence of Rooibos on the steroid output of H295R cells.

CHAPTER 4

The influence of Rooibos (*Aspalathus linearis*) on adrenal cytochrome P450 enzymes

4.1 Introduction

Cytochrome P450 enzymes are primarily responsible for detoxification, biotransformation and the removal of foreign compounds (xenobiotics) in mammals. In addition, P450-dependent enzymes are also involved in the biosynthesis of steroid hormones, which are important for the endocrine system, as discussed in Chapter 3. Although each steroidogenic enzyme can catalyse multiple reactions, often with different steroid substrates, these P450-dependent enzymes are highly specific to the steroid biosynthetic pathways (Estabrook 2005). As discussed earlier, the arrangement of the heme pigment in the active site of cytochrome P450-dependent enzymes bestows unique spectral properties on these hemoproteins. The binding of substrates and inhibitors to P450-dependent enzymes can thus be studied using difference spectroscopy, as discussed in section 4.2.6.1.

Rooibos, which is a good dietary source of flavonoids, is anecdotally reported to alleviate a variety of endocrine-related diseases, such as stress, hypertension, anxiety, depression and diabetes (Persson 2012; Schloms et al. 2013). Scientifically based assays, however, are needed to validate the anecdotal reports of Rooibos alleviating endocrine-related diseases. Most of the endocrine-related diseases are characterised by elevated levels of a particular steroid synthesised in the adrenal gland by the P450 enzymes. It is possible that Rooibos can modulate adrenal steroidogenesis, thus influencing the production of steroids in the adrenal, as it contains polyphenols that have been reported to affect steroidogenesis in H295R cells (Schloms et al. 2012; Hasegawa et al. 2013). Rooibos, and some flavonoids found in Rooibos, have been reported by Schloms et al. (2012) to affect adrenal cytochrome P450 enzymes in H295R cells, and in transfected COS-1 cells. The usage of reliable scientifically based assays, however, is necessary to validate the anecdotal reports of Rooibos alleviating endocrine-related diseases.

In this study, the effect of Rooibos (unfermented and fermented), and the flavonoids aspalathin, rutin and quercetin, on the binding of natural substrates (PROG and DOC) to

adrenal P450-dependent enzymes was investigated. DOC is a natural substrate of CYP11B1 and CYP11B2, while PROG is a natural substrate of CYP17A1 and CYP21A2. These enzymes play crucial roles in the biosynthesis of ALDO and cortisol, which are steroids that are important for the maintenance of homeostasis in the body, as mentioned earlier in section 3.6 of Chapter 3.

4.1.1 Methodology

The investigation employed the unique spectral properties of the P450-dependent enzymes by performing spectral substrate binding assays. The binding of PROG and DOC to microsomal and mitochondrial P450 enzymes was investigated in ovine adrenal preparations in the presence of the Rooibos extracts or selected flavonoids.

4.2. Materials and methods

4.2.1 Reagents

Dried unfermented and fermented Rooibos was provided by the South African Rooibos Council. Fermented and unfermented Rooibos are products of the Rooibos plant, obtained after harsh and mild processing conditions respectively. These conditions are oxidative and affect the flavonoid content of Rooibos (Joubert et al. 2008). The selected flavonoids (aspalathin, rutin and quercetin) and steroids (PROG and DOC) were purchased from Sigma Chemical Co (South Africa). Sheep adrenals were obtained from Tomis Abattoir in Hermon, Western Cape, South Africa. The adrenals were surgically removed and covered in ice immediately after removal. NADPH and sodium dithionate were purchased from Roche Diagnostic (South Africa). Pierce BCA^{TM} protein determination kits were purchased from Pierce (Rockford, IL, USA). A Varian Cary 100 double-beam UV-Vis spectrophotometer was used to record the changes in wavelengths in the presence and absence of Rooibos extracts.

4.2.2. Preparation of unfermented and fermented Rooibos extracts.

Methanolic extracts of Rooibos were prepared by placing 20 g dried Rooibos plant material in a glass soxhlet extractor. The extractor was fitted with a double walled condenser and placed on a round bottom flask containing 200 mL of chloroform. The extraction was done in

an air extraction hood for 8 hr at ± 60 ºC on a heating mantle. The chloroform phase was discarded after 8 hr and replaced with 200 mL of methanol for an additional 8 hr. During the time of extraction, the entire extraction setup was covered in aluminium foil to protect the flavonoids in the Rooibos against light degradation. The final extract of rooibos was removed and dried on a rotary evaporator under reduced pressure at 37 ºC. During the release of the vacuum from the dried extract, nitrogen gas was introduced to minimise oxidation. The round bottom flask was weighed before and after the extraction to obtain the mass of the Rooibos extract. The measured Rooibos extract was redissolved in deionised water to make a final concentration of 100 mg/mL and clarified by centrifugation at 6000 xg for 5 min. Aliquots (1 mL), prepared from the supernatant, were stored at -20 °C in dark conditions to avoid any compositional changes. Extractions were repeated for both the unfermented and fermented Rooibos. The Agricultural Research Council subsequently analysed the unfermented and fermented Rooibos extracts for flavonoid content using high-performance liquid chromatography with photodiode array detection (HPLC-DAD) method, as previously published by Beelders et al. (2004).

4.2.3 Preparation of ovine adrenal mitochondria and microsomes.

Ovine mitochondrial and microsomal preparations were obtained by differential centrifugation of chilled surgically removed adrenals from slaughtered ovine according to the method described by Yang and Cederbaum (1994). All procedures were performed in a temperature controlled room at 4 °C. The adrenals were defatted and decapsulated using a scalpel and washed with a 15 mM KCl solution. The adrenal tissue (101.09 g) was homogenised in 300 mL 10 mM Tris-HCl buffer (pH 7.4), Buffer A, using a Potter-Elvejhem glass homogeniser. Buffer A contained 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged for 15 min at 12 000 xg at 4 ºC to acquire a pellet, which contained the mitochondria, and the supernatant, which contained the microsomes.

The preparation of the mitochondria and microsomes is shown schematically in Figure 4.1. The mitochondrial pellet was resuspended in 200 mL of 10 mM Tris-HCl buffer (pH 7.4), Buffer C. Buffer C contained 1 mM EDTA, 0.25 M sucrose and 1 % bovine serum albumin (BSA) mixture. Buffer C subsequently was used to homogenise and centrifuge the mitochondrial pellet at 12000 x g for 15 min at 4 ºC. The supernatant was discarded and the

resulting pellet was resuspended in 200 mL of 10 mM Tris-HCl buffer (pH 7.4), Buffer B. Buffer B contained 150 mM KCl and 1 mM EDTA and was used to wash and homogenise the pellet, which subsequently was centrifuged at 12000 xg for 15 min at 4 ºC. The resulting supernatant was discarded. This washing procedure was repeated twice to remove fat and blood. The washed mitochondrial pellet was lyophilised overnight. To remove excess fat from the dried mitochondrial pellet, pure filtered dry acetone was used to suspend, homogenise and centrifuge the dry pellet at 12000 xg for 10 min at 4 ºC. The defatted mitochondrial pellet was lyophilised overnight. The dry mitochondrial preparation was stored at -20 °C for future use.

The microsomal preparation was prepared by slowly stirring PEG 8000 (50 % w/v) into the supernatant containing the microsomes to a final concentration of 8.5 %. The mixture was stirred for 10 min and then centrifuged at 13000 xg for 20 min. The supernatant was discarded and the microsomal pellet was resuspended in 10 mL of Buffer B. This step was repeated until the supernatant was clear (at least three times). The final microsomal pellet was resuspended in 15 mL of Buffer A. The suspension was stored at -80 °C in 1 mL aliquots for future use.

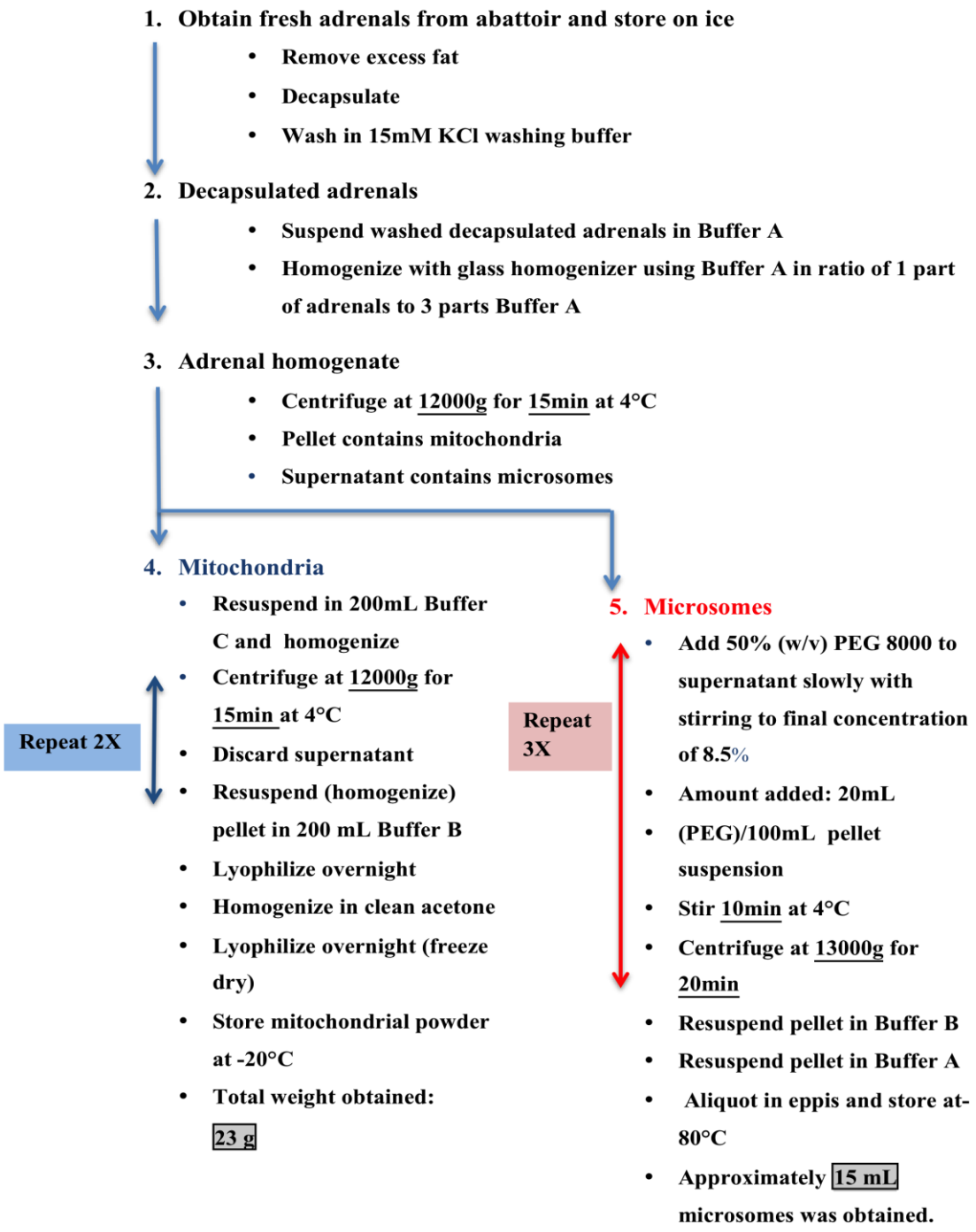


Figure 4.1. Flow diagram showing the preparation of ovine mitochondrial and microsomal preparations.

4.2.4 Determination of cytochrome P450 concentration

The cytochrome P450 content of the adrenal mitochondrial and microsomal preparations was determined from a type II difference spectrum using the carbon monoxide (CO) method as described by Omura and Sato (1964). The spectrum was allowed to develop fully for at least

5 min, as the reduction of the CO cytochrome P450 by sodium dithionite (a strong reducing agent) elicits a slow reaction. For this investigation, the mitochondria powder (2 mg/mL) was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 10 % ethylene glycol. The mitochondrial suspension was sonicated on ice for 5 min at one-min intervals. To determine the microsomal P450 concentration, the microsomal preparation was diluted at a ratio of 1:3 of microsomal preparation and Buffer A.

The 4 mL mitochondrial preparation sonicate was saturated with carbon monoxide, and 1.5 mL of the saturated sonicate was subsequently added to each of two optically matched quartz cuvettes, the sample and reference cuvette respectively. A baseline was recorded between 400 and 500 nm. Sodium dithionate (~2 µg) was subsequently added to the sample cuvette alone and the contents were mixed by gentle inversion. The reference cuvette was also mixed by gentle inversion to ensure homogeneity. The spectrum was recorded between 400 and 500 nm after 10 min. The mitochondrial sonicate in the reference cuvette served as a baseline for the experiment. The same procedure was carried out for the microsomal preparations. The cytochrome P450 concentration (C) of the preparations was determined using the Beer-Lambert law presented in Eq. 4.1.

$$\Delta Abs = \varepsilon Cl \qquad \text{(Eq. 4.1)}$$

where the ΔAbs is the difference in absorbance between 450 nm and 500 nm, the molar extinction coefficient (ε) = 91 cm-1 mM-1, and l is the path length through the solution (1 cm) as described by Omura and Sato (1994).

4.2.5 Protein determination

A Pierce BCA™ protein assay kit was used to determine the protein concentration of the preparations. Phosphate buffer (1 mM) was used as a blank and for the dilution of the samples. A series of standard protein concentrations was prepared: 0.25, 125, 250, 500, 750, 1 000, 1 500 and 2 000 µg/mL of the BSA in 1 mM phosphate buffer. Working reagent was mixed according to the manufacturer's protocol. Only 200 µL of the working reagent was used per well, containing only 10 µL of the mitochondrial sonicate or 10 µL of the microsomal dilution or 10 µL of each standard sample in a 96-well micro titre plate. The plate was incubated at 37 °C for 30 min in order to obtain the visible colour, and absorbance was

recorded at 545 nm. The protein determination was carried out in triplicate. A standard curve was constructed from the standard sample results and was used to determine the protein concentration.

4.2.6. Bioactivity in spectral binding assay

PROG was used as substrate for the microsomal P450 enzymes, whereas DOC was used as substrate for the mitochondrial P450 enzymes. The substrates were used at saturating concentrations of 13 μM and 12 μM for PROG and DOC respectively. To investigate the interference of Rooibos or selected flavonoids on the binding of the substrates to the respective P450 enzymes, varying concentrations of Rooibos extracts and flavonoids were used. The selected flavonoids were dissolved in ethanol.

4.2.6.1. Substrate-induced difference spectra

The interaction of the Rooibos extracts with the P450 enzymes was investigated at room temperature using UV-Vis spectroscopy. The binding of PROG to CYP17A1 and CYP21A2 in a microsomal preparation, and the binding of DOC to CYP11B1 and CYP11B2 in a mitochondrial preparation, were assayed in the presence of the different concentrations of unfermented and fermented Rooibos extracts. A similar investigation was carried out using different concentrations of the selected flavonoids dissolved in ethanol. The microsomal preparation was pipetted into two optically matched quartz cuvettes to record a baseline absorbance. The two quartz cuvettes represent the reference and the sample.

For the microsomal P450 substrate binding investigation, PROG, dissolved in ethanol, and ethanol were added to the sample and reference cuvettes respectively at equal volumes, in the presence of Rooibos extracts or flavonoids. The cuvettes were gently inverted and a substrate-induced difference spectrum was recorded. To investigate the percentage inhibition, different concentrations of Rooibos extracts and flavonoids were used. Compound interference with PROG binding to P450 enzymes in microsomal preparation was indicated by a reduction in the amplitude of the peak and trough of the type I difference spectrum (see Figure 4.2). The interference is illustrated by the decrease in the absorbance maximum at 390 nm and an increase in the absorbance minimum at 420 nm when compared to the substrate-

induced difference spectra produced in the absence of the compounds. A similar investigation was also carried out for DOC binding to P450 enzymes in mitochondrial preparations.

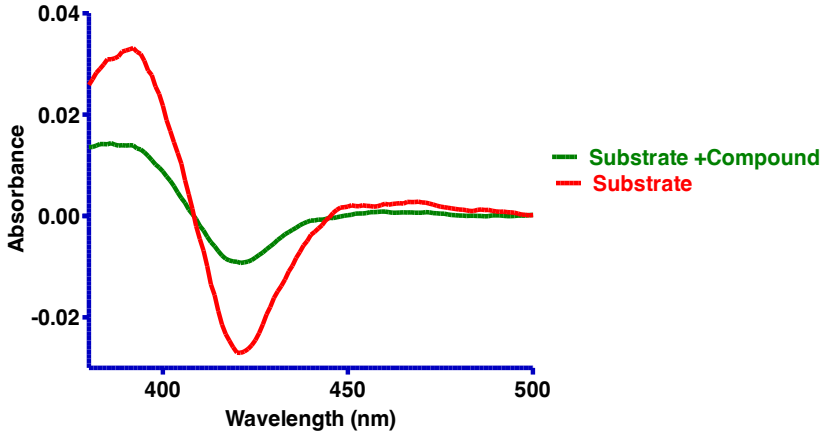


Figure 4.2. Example of type I substrate-induced difference spectra. PROG (13 µM) was used as substrate binding to ovine microsomal P450 enzymes in the absence and presence of rutin (26 µM). The P450 concentration was 0.5 nmol/mg protein.

The interaction of the compounds with the P450 enzymes was expressed in percentage inhibition, as shown below:

$$\% \text{ Inhibition} = 100 - ((I/C) \text{ X } 100) \qquad \text{(Eq. 4.2)}$$

where I = absorbance at 390 nm minus absorbance at 420 nm in the presence of inhibitor, and C = absorbance at 390 nm minus absorbance at 420 nm in the absence of inhibitor.

4.2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 5.0 for Windows (GraphPad Software), San Diego, California, USA. Binding assays were expressed as the mean, with error bars representing the standard error of the mean (SEM). Statistics of the binding assays were calculated by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Values of $P < 0.05$ and values of $P < 0.0001$ of one-way ANOVA and Dunnett's multiple comparison test respectively were considered to be statistically significant. Protein concentration analyses were done using Microsoft® Excel 2003, based on a standard curve, in which case the results were expressed as µg protein/mL.

4.3. Results

4.3.1 Analysis of Rooibos extracts

Extracts of the unfermented and fermented Rooibos used for the binding assays were analysed for flavonoid quantification using HPLC-DAD, as previously done by Beelders et al. (2004). The results showed that the unfermented Rooibos extracts had higher levels of dihydrochalcones relative to the other flavonoid levels. Within the dihydrochalcone group, the concentration of aspalathin was 5.7-fold higher than nothofagin. The unfermented Rooibos extract's flavone content showed that orientin and isoorientin levels were roughly 5-fold higher than isovitexin and vitexin. The unfermented Rooibos extract's flavonol concentration showed that quercetin-3-*O*-rutinoside was roughly 2-fold the level of rutin. The fermented Rooibos extract's flavones, particularly orientin and isoorientin, were the most abundant flavonoids in the extract, although isovitexin and vitexin levels were roughly 5-fold lower than isoorientin and orientin. Quercetin-3-*O*-rutinoside's level was 5-fold higher than rutin in the fermented extract's flavonol group. The fermented extract's dihydrochalcones showed that the nothofagin level was 3.5-fold lower than that of aspalathin.

When comparing the flavonoid levels of both extracts (Table 4.1), the unfermented extract demonstrated a higher level of dihydrochalcones. Aspalathin and nothofagin levels were 28-fold and 19-fold higher respectively in the unfermented extracts than in the fermented extracts. The quercetin-3-O-rutinoside level was similar in both extracts, whereas rutin was 4-fold higher in the unfermented extract than in the fermented extract. The flavones of unfermented and fermented Rooibos extracts were similar in level.

Table 4.1. Flavonoid compounds (µg) present in the fermented and unfermented Rooibos extracts. Flavonoids were extracted from 1 g dried Rooibos plant material. Results are expressed as the mean ± SEM.

Flavonoids	Unfermented extract	Fermented extract
	ug	ug
Aspalathin	20556.7 ± 211.9	735.8 ± 10.9
Nothofagin	3596.8 ± 35.1	186.2 ± 4.5
Rutin	542.1 ± 5.6	162.8 ±1.9
Quercetin-3-*O*-rutinoside	986.2 ± 12.0	1000.8 ±13.8
Orientin	2196.6 ± 27.8	2158.2 ± 31.2
Isoorientin	2863 ± 39.4	2524.7 ± 37.3
Vitexin	424.7 ± 3.3	466.0 ± 3.9
Isovitexin	524.9 ± 5.1	485.5 ± 4.3

4.3.2 Determination of cytochrome P450 enzyme concentration.

The cytochrome P450 concentrations of the ovine adrenal preparations were determined by carbon monoxide induced difference spectra as demonstrated in Figure 4.3, (Omura & Sato 1964). The sodium dithionate reduced carbon monoxide spectrum is formed from a slow reaction, therefore allowing the absorbance at 450 nm to fully develop.

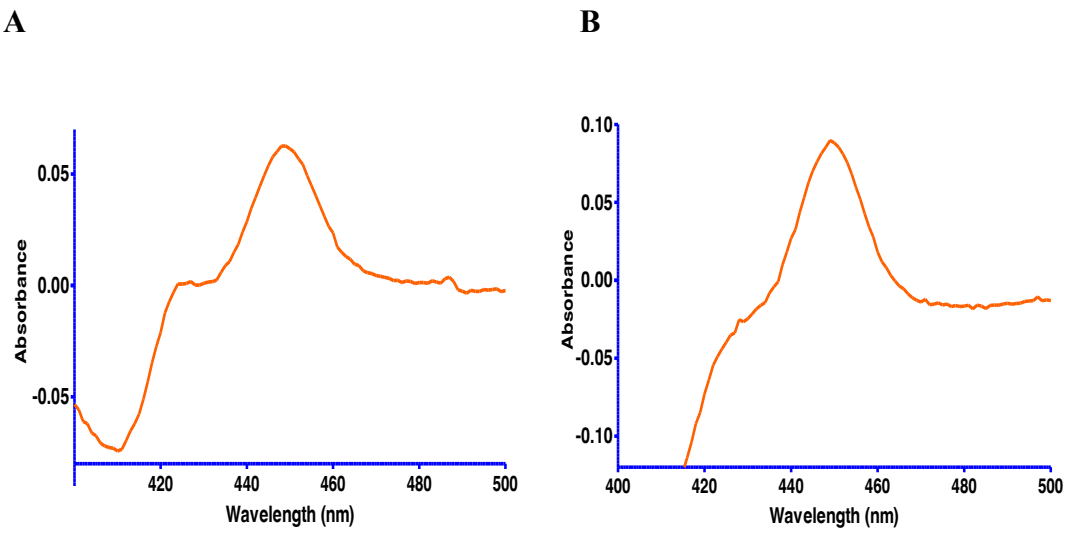


Figure 4.3. Type II carbon monoxide induced difference spectra. Sodium dithionate reduced spectra of A) mitochondrial preparation, B) microsomal preparation. Mitochondrial [P450] = 0.7 µM (0.7 nmol/mg protein) and microsomal [P450] = 1 µM (0.5 nmol/mg protein).

4.3.4 Substrate binding assays

The binding assay activity on the ovine adrenal preparations was investigated to determine the interference of Rooibos extracts and selected flavonoids (aspalathin, rutin and quercetin) in substrate binding to adrenal P450 enzymes. This investigation was based on the unique spectral properties of the P450 enzymes. P450 enzymes are heme proteins, since they contain a ferric iron. The heme iron's equilibrium changes between a high and a low spin state in the presence of an appropriate substrate in the active site, as mentioned in section 3.4 of Chapter 3. The high and low spin states of the heme iron have profoundly different spectra, making it possible to easily monitor the substrate binding spectroscopically. The change in the spin state forms the basis of the type I spectrum. The spectrum has a peak maximum at 390 nm and a peak minimum at 420 nm. Any compounds that interfere with the binding of substrate to the P450 enzymes reduce the amplitude of the spectrum (viz. a reduced peak maximum and increased peak minimum), as shown in Figure 4.2.

Adrenal mitochondrial preparation contains CYP11A1, CYP11B1 and CYP11B2, whilst the microsomal preparation contains CYP17A1, CYP21A2 and 3βHSD steroidogenic enzymes (Werck-reichhart & Feyereisen 2000). DOC was chosen as substrate for the mitochondrial preparation, as it is the substrate for CYP11B1 and CYP11B2, whilst PROG was chosen as the substrate for the microsomal preparation, as it is a substrate for CYP17A1 and CYP21A2.

These substrates induce a type I spectrum when they bind to the respective P450 enzymes in the presence of varying concentrations of either the Rooibos extracts or the selected flavonoids (Table 4.2 and Table 4.3). The concentrations of Rooibos extracts that were used for this study ranged from 0.2 mg/ml to 1 mg/ml for unfermented extracts and 0.2 mg/ml to 2 mg/ml for fermented extracts, while for the flavonoids the concentration ranged from 4 μM to 53 μM. Substrate binding interference was indicated by the reduction in the amplitude of the spectrum (see Figure 4.2). Only the concentrations of the extracts and the flavonoids that showed significant inhibitions were recorded. The flavonoid content of the extract concentration that affected substrate binding significantly was determined (see Table 4.4).

The results of the substrate binding studies suggest that the unfermented extract inhibited the binding of PROG to the P450 enzymes in the microsomal preparation more than that of the fermented Rooibos extract. The unfermented Rooibos extract inhibited the binding more than the fermented Rooibos extract when both extracts were used at a concentration of between 0.2 mg/mL and 0.7 mg/mL (Figure 4.4 A). The fermented Rooibos extract caused a 7.1 % inhibition, whilst the unfermented Rooibos extract caused a 21.5 % inhibition. In the mitochondrial preparation, in Figure 4.4 B, the results show a similar degree of inhibition, where the fermented and unfermented Rooibos extracts inhibited DOC binding by 41 % and 48 % respectively. When comparing the degree of substrate binding inhibition by the extracts at the same concentration for the microsomal and mitochondrial P450 enzymes, it is observed that the unfermented Rooibos extract interfered more with substrate binding than the fermented Rooibos extract. Also, the results suggest that the fermented extract inhibits substrate binding to P450 enzymes in mitochondrial preparations about 5.8-fold more than in microsomal preparations, while unfermented Rooibos extract inhibits substrate binding to P450 enzymes about 2-fold more in mitochondrial preparations compared to the microsomal preparations.

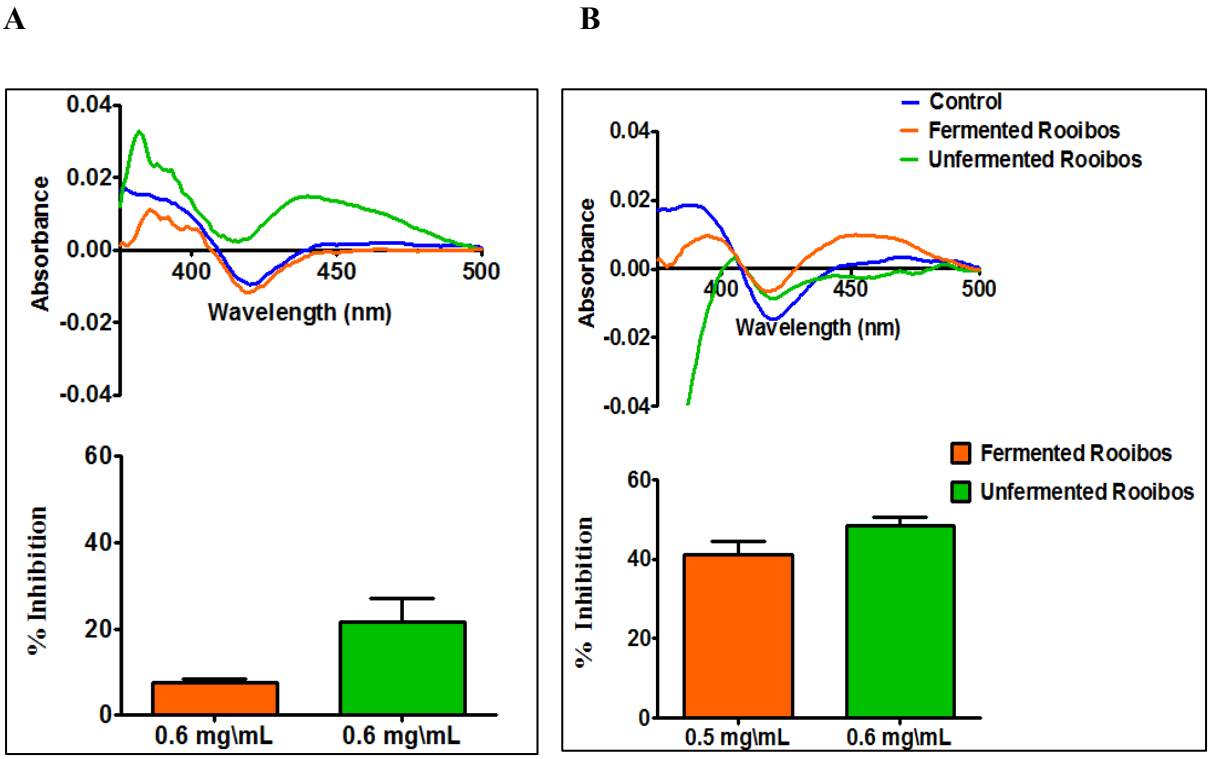


Figure 4.4. Effect of Rooibos extracts on substrate binding to microsomal and mitochondrial P450 enzymes. Type 1 difference spectra and percentage inhibition of A) PROG (12 µM) binding to ovine microsomal P450 enzymes (0.5 nmol/mg protein), B) DOC (13 µM) binding to ovine mitochondrial P450 enzymes (0.7 nmol/mg protein) in the presence and absence of unfermented and fermented Rooibos extracts. Results were determined by a one way ANOVA, followed by Dunnett's multiple comparison test. Only the concentrations that showed significant inhibition values are shown and the data is representative of 3 independent measurements. Results are presented as the mean error bars present ± SEM, and n = 3.

At 0.6 mg/mL Rooibos extracts inhibited substrate binding to P450 enzymes in both the microsomal and mitochondrial preparations. Overall, Rooibos extracts appeared to interfere with substrate binding to P450 enzymes in mitochondrial preparations to a greater extent than in microsomal preparations at similar concentrations.

Table 4.2. Column statistics illustrating inhibition of PROG (13 µM) and DOC (12 µM) binding P450 enzymes in A) microsomal preparation (0.5 nmol/mg protein) and B) mitochondrial preparation (0.7 nmol/mg protein) respectively in the absence and presence of increasing Rooibos extract concentrations (One way ANOVA, followed by Dunnett's Multiple Comparison Test, then column statistics (P < 0.001 = ***, P< 0.01= **)).

Compound	Microsomal preparation				Mitochondrial preparation			
	Concentration (µM)	Abs	Mean	± Std (P < 0.001)	Concentration (µM)	Abs	Mean	± Std (P < 0.001)
Steroid	13	0.036	0.036	0.001	12	0.040	0.040	0.020
Unfermented Rooibos	0.6	0.029	0.029	0.003 **	0.6	0.018	0.018	0.020 ***
Fermented Rooibos	0.6	0.032	0.032	0.001	0.5	0.019	0.019	0.020 **

At 13 µM, rutin inhibited PROG binding to P450 enzymes in microsomal preparations by 19 % percent, whereas a four times greater concentration of quercetin resulted in a similar degree of inhibition (see Figure 4.5 A). In mitochondrial preparations a 60.5 % inhibition, at a concentration of 18 µM rutin, was observed, while a similar degree of inhibition (65 %) was observed when double the concentration of quercetin was used (see Figure 4.5 B). The data obtained shows that lower concentrations of both rutin and quercetin resulted in a higher degree of inhibition in the mitochondrial preparations than in the microsomal preparations, and that substrate binding to P450 enzymes was inhibited more in the mitochondrial preparations than in the microsomal preparations. Aspalathin (results not shown because they were not statistically significant) did not interfere with substrate binding to the P450 enzymes in both the mitochondrial and microsomal preparations.

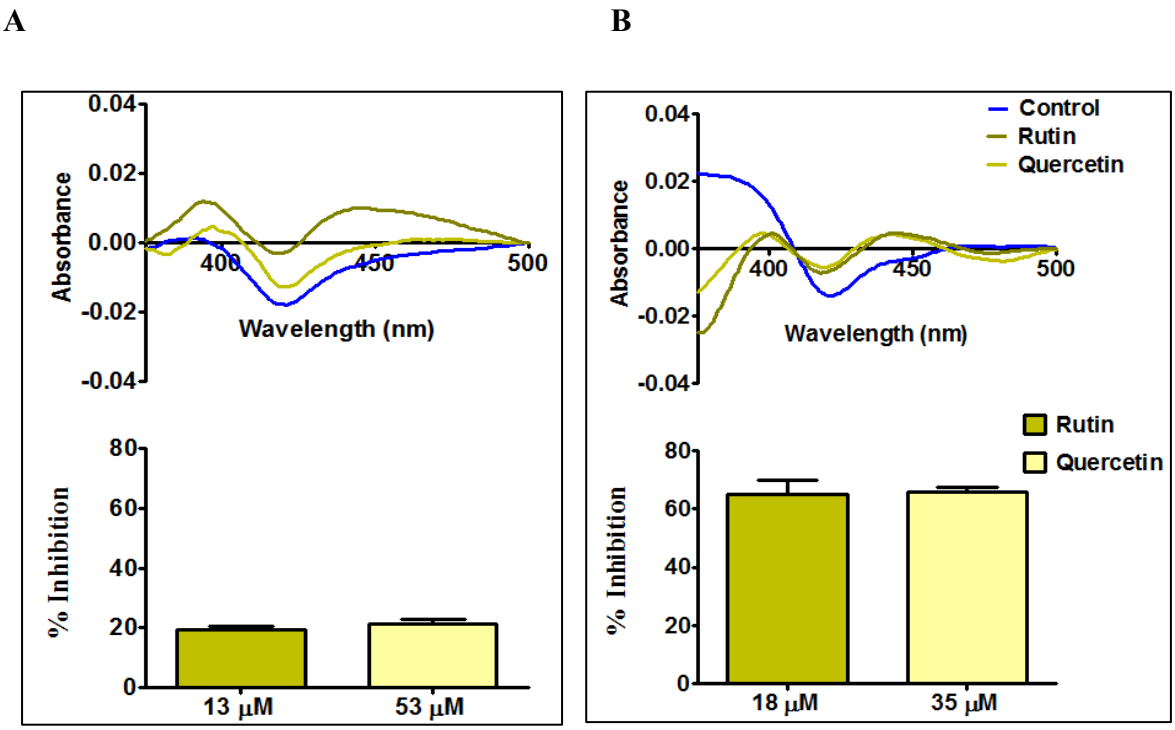


Figure 4.5. Effect of flavonoids on substrate binding to microsomal and mitochondrial P450 enzymes. Induced Type 1 difference spectra and percentage inhibition of, A) PROG (12 µM) binding to ovine microsomal P450 enzymes (0.5 nmol/mg protein), B) DOC (13 µM) binding to ovine mitochondrial P450 enzymes (0.7 nmol/mg protein) in the presence and absence of selected flavonoids. Results were determined by a One way ANOVA, followed by Dunnett's multiple comparison test. Only the concentrations that showed significant inhibition values are shown and the data is representative of 3 independent measurements. Results are presented as the mean error bars present ± SEM, and n = 3.

Statistical analysis of substrate binding inhibition in the presence of the selected flavonoids showed that, with an increase in concentration of both rutin and quercetin, substrate binding to the P450 enzymes in both the mitochondrial and microsomal preparations was inhibited (see Table 4.3). The data summarised in Table 4.3 show that the flavonoids interfered with substrate binding to the P450 enzymes more in the mitochondrial preparations than in the microsomal preparations.

Table 4.3. Column statistics illustrating inhibition of PROG (13 µM) and DOC (12 µM) binding P450 enzymes in A) microsomal preparation (0.5 nmol/mg protein) and B) mitochondrial preparation (0.7 nmol/mg protein) respectively in the absence and presence of increasing concentrations of the selected flavonoid concentrations (One way ANOVA, followed by Dunnett's Multiple Comparison Test then column statistics ($P < 0.001$ = ***, $P < 0.005$ = *)).

Compound	Microsomal preparation				Mitochondrial preparation			
	Concentration (µM)	Abs	Mean	± Std ($P < 0.001$)	Concentration (µM)	Abs	Mean	± Std ($P < 0.001$)
Steroid	13	0.036	0.036	0.001	12	0.040	0.040	0.020
Rutin	6.6	0.021	0.022	0.002 ***	4.4	0.025	0.025	0.004 ***
	20	0.019	0.019	0.002 ***	13	0.024	0.024	0.002 ***
	26	0.016	0.016	0.001 ***	18	0.014	0.014	0.003 ***
Quercetin	40	0.026	0.026	0.001 *	18	0.023	0.023	0.002 ***
	53	0.023	0.023	0.001 ***	35	0.014	0.014	0.002 ***

The flavonoid levels in Rooibos extracts that significantly inhibited the binding of substrates to microsomal and mitochondrial P450 enzymes were determined (see Table 4.4). At a concentration of 0.6 mg/mL, unfermented Rooibos extract had higher levels of dihydrochalcones compared to flavone and flavonols, where aspalathin was 5-fold higher than the nothofagin. The flavones, orientin and isoorientin, were 4-fold higher than vitexin and isovitexin. For the flavonols, the quercetin-3-*O*-rutinoside concentration was significantly higher than the rutin concentration. Fermented Rooibos extract had similar levels of flavonoids to unfermented Rooibos extract at concentrations of 0.5 mg/ml and 0.6 mg/ml respectively. The fermented extract had higher levels of flavones, in particular orientin and isoorientin, which were double the levels of quercetin-3-*O*-robinobioside. The level of quercetin-3-*O*-robinobioside was 5.6-fold higher than that of rutin. The nothofagin level was fourfold lower than that of aspalathin. The flavones were generally present in higher levels than the other flavonoids.

Table 4.4. Flavonoid compounds (µg) present in the Rooibos extract added to microsomal and mitochondrial preparations. Results are expressed as the mean ± SEM.

Flavonoids (µg)	Microsomal preparation		Mitochondrial preparation	
	Unfermented extract (0.6 mg/mL)	Fermented extract (0.6 mg/mL)	Unfermented extract (0.6 mg/mL)	Fermented extract (0.5 mg/mL)
Aspalathin	78.1 ± 0.8	2.0 ± 0.0	78.1 ± 0.8	2.3 ± 0.0
Nothofagin	13.7 ± 0.1	0.5 ± 0.0	13.7 ± 0.1	0.6 ± 0.0
Rutin	2.1 ± 0.0	0.5 ± 0.0	2.1 ± 0.0	0.5 ± 0.0
Quercetin-3-*O*-rutinoside	3.7 ± 0.0	2.8 ± 0.0	3.7 ± 0.0	3.2 ± 0.0
Orientin	8.3 ± 0.1	6.1 ± 0.1	8.3 ± 0.1	6.8 ± 0.1
Isoorientin	10.9 ± 0.1	7.0 ± 0.1	10.9 ± 0.1	8.0 ± 0.1
Vitexin	1.6 ± 0.0	1.0 ± 0.0	1.6 ± 0.0	1.5 ± 0.0
Isovitexin	2.0 ± 0.0	1.4 ± 0.0	2.0 ± 0.0	1.5 ± 0.0

4.4. Discussion

Prior to conducting the substrate binding assays, major flavonoids present in the Rooibos extracts were analysed. The analyses showed that the unfermented extract contained higher levels of aspalathin, nothofagin and rutin than the fermented extract. Also, these flavonoids were present in higher levels in both extracts compared to other flavonoids (see Table 4.5). In contrast to the unfermented extract flavonoid levels, the fermented extract contained relatively high levels of flavones (isoorientin and orientin) and quercetin-3-O-robinobioside compared to the other flavonoids. The flavonoid levels in the unfermented extract were similar to the flavonoid content of the unfermented extract as reported by Schloms et al. (2013). However, the flavone levels of both extracts were similar. In the flavonol group, rutin was 3.3-fold more abundant in the unfermented extract than in the fermented extract.

Previous studies conducted in our laboratory have shown that the aqueous and methanolic extracts of unfermented Rooibos inhibited the binding of PROG to microsomal P450 enzymes to a greater extent than the aqueous and methanolic extracts of fermented Rooibos. Due to this observation, no further investigations were done in the presence of fermented Rooibos extracts. Interestingly, however, in the studies conducted by Perold (2009) it was shown that the aqueous extracts of unfermented Rooibos inhibited PROG binding to P450 enzymes in the microsomal preparations more than in the methanolic unfermented extracts (Perold 2009). In the current study it was observed that both the methanolic extracts of Rooibos interfered with substrate binding to P450 enzymes in the microsomal and mitochondrial preparations.

The investigation has shown that PROG is inhibited from binding to microsomal steroidogenic enzymes by unfermented extract to a higher degree than by the fermented Rooibos extract at the same concentration (0.6 mg/mL). These results corroborate previous findings by Perold (2009) on the effect of methanolic Rooibos extracts on microsomal P450 enzymes. In this study it was found that methanolic extracts of unfermented Rooibos inhibited the binding of PROG to microsomal enzymes to greater extent than the methanolic extract of fermented Rooibos (Perold 2009). This could be attributed to the high levels of flavonoids found in unfermented extracts, which could have competitively inhibited the binding of PROG (see Table 4.5). However, in the mitochondrial preparation, DOC binding

was inhibited to the same degree (roughly 60 %) and at a similar concentration of both Rooibos extracts (0.5 to 0.6 mg/mL). This could be due to the quercetin-3-O-robinobioside and flavone levels being similar in both extracts (see Table 4.4) and having a combined inhibitory effect on the substrate binding to the P450 enzymes.

Since flavonoids have been reported to modulate P450 enzyme activity, in particular CYP17A1 and CYP21A2 (Buters 1997; Ohno et al. 2002), flavonoids were used to determine the interference with substrate binding in the mitochondrial and microsomal preparations. Flavonols (rutin and quercetin) and aspalathin were used for this investigation. The flavonols inhibited the substrate binding in both the preparations in a dose-dependent manner (Table 4.3). Low concentrations of rutin (13 µM and 18 µM) inhibited the microsomal and mitochondrial P450 enzymes more than quercetin, which was used at higher concentrations (53 µM and 35 µM). These findings suggest that rutin is more potent than quercetin, as it results in the inhibition of substrate binding to P450 enzymes at lower concentrations than quercetin. It can be speculated that rutin is able to form a stable complex that binds in the active site of the P450 enzymes. Moreover, it is assumed that the substrate binding interference caused by rutin and quercetin is due to the hydroxyl groups at the 6^{th} position of their pyran ring and 4^{th} position on their benzene ring. Ohno et al. (2002) reported this assumption to be the cause of the inhibition of the production of cortisol in H295R cells. Varying concentrations of aspalathin were found not to inhibit substrate binding (results not shown). Aspalathin has been reported by Schloms et al. (2012) to affect P450 enzyme activity (mechanism unknown), it is also possible that higher concentrations of aspalathin, such as that found in Rooibos, could interfere with substrate binding to P450 enzymes.

4.5. Conclusion

The substrate binding assays carried out showed that unfermented and fermented Rooibos and flavones (quercetin and rutin) interfere with substrate binding to adrenal P450 enzymes, and that aspalathin does not interfere with substrate binding. Rutin is more potent than quercetin; therefore it is possible that the inhibition seen with Rooibos extract could be due mainly to the presence of rutin.

Although the investigations described above show the interference of Rooibos and flavonoids with substrates binding to the enzymes, these assays do not reflect their influence on the

catalytic activity of the specific P450 enzymes. Consequently, substrate conversion assays were conducted to determine the influence of Rooibos and flavonoids on the catalytic activities of the enzymes.

CHAPTER 5

The influence of Rooibos (*Aspalathus linearis*) on adrenal steroidogenesis

5.1. Introduction

The endocrine system is responsible for the regulation and maintenance of physiological functions. The system comprises various endocrine glands that include the pituitary gland, ovaries, testes, thyroid gland and the adrenal gland. The endocrine glands produce hormones that are messengers secreted to act on target tissues or organs to regulate physiological functions. The adrenal gland is a complex organ involved in the biosynthesis and secretion of steroids by specific enzymes, which were as described in Chapter 3. Steroidogenic enzymes (P450 enzymes and HSDs) are biological catalysts involved in the biosynthesis of steroids (glucocorticoids, mineralocorticoids and androgens). Steroids control many physiological processes via endocrine signalling pathways, and contribute to the transcriptional regulation of P450 enzymes. As discussed in Chapter 3, the steroids are synthesised from cholesterol and are transported via diffusion or the blood stream to elicit their biological activities.

The regulation of steroidogenesis generally involves the HPA axis and the RAAS, which are examples of endocrine feedback loops regulated by circulating steroids. Any changes in steroidogenic enzyme activity can alter steroid metabolism and, consequently, the concentrations of circulating plasma steroids. The alteration of the circulating steroids can lead to an imbalance of water and mineral homeostasis, glucose homeostasis and sexual development, thus leading to clinical conditions. Inadequate levels of mineralocorticoids, mainly ALDO, and glucocorticoids, mainly cortisol, have been reported to be associated with most of the endocrine diseases such as hypertension, diabetes, stress and anxiety.

The consumption of Rooibos herbal infusions has been reported to aid endocrine related illnesses that are linked to abnormal levels of adrenal steroids, as mentioned in Chapter 2, section 2.6. Some studies have shown that fermented Rooibos has the ability to inhibit the activity of ACE, an important enzyme involved in the RAAS activity, in human volunteers (Persson et al. 2010). The effects of Rooibos on human health, including anti-mutagenic, anti-fungal and anti-oxidant capacity, have been investigated and validated by various studies mentioned in Chapter 2, but its influence on the endocrine system is not widely reported. In a

study done by Schloms et al. (2012), they observed a decrease in total steroids produced in H295R cells in the presence of methanolic extracts of unfermented Rooibos under stressed or stimulated conditions. In the same investigation it was shown that CYP17A1 and CYP21A2, expressed in COS-1 cells, were inhibited by 10 µM dihydrochalcones (asphalathin and nothofagin) (Schloms et al. 2012). These studies show that unfermented Rooibos have a direct effect on the P450 enzymes catalysing steroidogenesis.

The main objective of this study was to investigate the influence of Rooibos and selected flavonoids on the catalytic activity of individual P450 enzymes found in the microsomes and mitochondria, charged with the primary function of catalysing adrenal steroid biosynthesis. The influence of Rooibos was also investigated in H295R cells, an adrenal cell model, to determine the effect on the overall steroidogenesic output. Not only will these investigations demonstrate the influence of methanolic extracts of Rooibos and selected flavonoids on P450 enzyme activity, but also its effect on the flux through the steroidogenesis pathway. These studies allow the comparison of the effect of unfermented and fermented Rooibos on individual P450 enzymes and the steroidogenic pathway, and the overall steroidogenesis assays done in H295R cells.

Substrate conversion assays were investigated in COS-1 cells in the presence and absence of methanolic extracts of Rooibos and selected flavonoids. COS-1 cells, a non-steroidogenic monkey kidney cell line, were transfected with expression vectors containing CYP17A1/pCI-neo and CYP21A2/pCI-neo, while expression vectors containing CYP11B1/pCMV and CYP11B2/pCMV were co-transfected with adrenodoxin enzyme (ADX) and the appropriate steroids were assayed. CYP17A1 and CYP21A2 enzymes represent the microsomal *in vitro* studies, while CYP11B1 and CYP11B2 enzymes represent the mitochondrial *in vitro* studies. These assays were followed by an investigation into the effects of methanolic extracts of Rooibos on steroid hormone production in a human adrenal cell line, H295R.

5.2. Materials and methods

5.2.1 Reagents

Rooibos and selected flavonoids (described in section 4.2.1) were also used in the following investigations. PREG, PROG, DOC and deoxycortisol were purchased from Sigma Chemical

Co. (St Louis, MO, USA). Dulbecco's modified Eagles's medium (DMEM), and forskolin were acquired from Sigma Chemical Co. Deuterated cortisol (D4-cortisol) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Trypan blue, a diazo dye, was purchased from InvitrogenTM Life Technologies (Paisley, UK) Penicillin-streptomycin, fetal calf serum and trypsin-EDTA were purchased from Gibco-BRL (Gaithersburg, MD, USA). Dulbecco's modified Eagles medium/F-12 and gentamicin were purchased from Invitrogen/Gibco (Grand Island, NY, USA). Comic calf serum was supplied by HyClone, Thermo Scientific (South Logan, UT, USA). Luria broth medium constituents (tryptone, yeast extract) were purchased from Difco Laboratories (Detroit, MI, USA). Nucleobond AX plasmid preparation kits were purchased from Macherey-Nagel (Duren, Germany).

COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Mirus TransITR-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). H295R cells were a gift from Professor W. E Rainey (Department of Obstetrics & Gynecology Biochemistry, and The Cecil H. & Ida Green Centre for Reproductive Biology Sciences, Southwestern Medical Centre (Dallas, USA)). The UPLC BEH column was purchased from Waters (Milford, MA, USA), and the Kinetex PFP column was purchased from Phenomenex (Torrance, CA, USA).

All other chemicals, of high quality, were purchased from reputable scientific chemical supply companies.

5.2.2 Preparation of methanolic extracts of unfermented and fermented Rooibos.

Rooibos extracts were prepared by the extraction of plant material using chloroform and methanol as described in section 4.2.2 (Chapter 4). The extracted Rooibos was dried and resuspended in deionised water (mg/ml). Aliquots were stored at -20 °C until further use. Flavonoids (2 mg/ml) and steroids (2 mg/ml) were dissolved in ethanol and stored at 4 °C.

5.2.3 Preparation of plasmid for transfection.

Luria broth medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) was innoculated with transformed *E. coli* stocks and incubated overnight at 37 °C. Plasmid vectors included pCI-neo containing Angora CYP17A1 (CYP17A1/pCI-neo), Angora CYP21A2 (CYP21A2/pCI-neo); pCMV containing human CYP11B1/pCMV and CYP11B2/pCMV; and pCD

containing human ADX (pCD-ADX). The cultures were harvested by centrifugation and plasmid DNA was isolated using a Nucleobond AX plasmid kit according to the manufacturer's instructions. The plasmids obtained were stored at -20 °C.

Agarose gel electrophoresis was used to determine the integrity of the isolated plasmids. DNA samples of 250 ng, were analysed on an agarose gel, 1 %, in TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA). DNA marker (1 Kb ladder, Promega) and 5 µL 6 X Orange DNA loading dye consisting of 10mM Tris- HCl (pH 7.6), 0.15 % orange G, 0.03 % xylene cyanol FF, 60 % glycerol and 60 mM EDTA was used.

5.2.4 Determination of steroid conversion and production in model cell sytems

The influence of Rooibos on steroid conversion and overall steroidogenesis was assayed in COS- 1 cells, a non-steroidogenic cell line, and H295R cells, a human adrenocortical tumour cell line respectively.

5.2.4.1 Culturing and maintenance of COS-1 cells

COS-1 cells are a cell line derived from kidney tissue of the African green monkey, stably transformed with an origin defective mutant of SV40 encoding the wild type T antigen. Frozen COS-1 cells (1 ml) were thawed, resuspended and grown in 10 ml sterile culture media (DMEM high glucose, containing 0.15 % NaHCO3, 10 % foetal calf serum and 1 % penicillin streptomycin) in a 100 mm tissue culture dish at 37 °C, 5 % CO_2, 90 %. The media was replaced with fresh media on a daily basis until the cells were visibly confluent under the microscope. The confluent cells were detached from the plate using trypsin-EDTA (0.25 % w/v) and re-suspended in 30 mL culture media.

Detaching the cells from a 100 mm dish plate involved gently aspirating the media from the cells, washing the cells – which had been incubated for 2 min in the presence of 1 mL trypsin-EDTA – with 0.5 mL trypsin-EDTA by gentle swirling, which were incubated for 2 min in the presence of 1mL trypsin-EDTA. Cells were resuspended in 5 mL media to stop trypsin-EDTA activity. The suspension was centrifuged at 500 ×g for 5 min and the supernatant was discarded. The cells were resuspended in 10 mL of media. At this stage the cells can be replated into three sterile 100 mm tissue culture dishes (10 mL/dish) and incubated for experimental investigations by further culturing or resuspending in freezing

media (culture medium containing 10 % DMSO), and then storing in cryovials at -80 °C for 48 hr before storage in liquid nitrogen for future use

For investigation purposes, confluent cells were replated into 12 well plates (2.5 cm^2) at 1 × 10^5 and incubated for 24 hr. A cell counter (Invitrogen Countess automated cell counter) was used to count the cells. Before counting the cells, 10 µL of the resuspended cells were gently but thoroughly mixed (to ensure homogeneity) with 10 µL of trypan blue, and 10 µL of this was used for counting. Trypan blue is derived from toluidine and not taken up by live cells. The method is also referred to as a dye exclusion method, and non-viable cells and viable cells are recorded. Viable cells are expressed as follows:

% viable cells = (1.00 – (Number of blue cells / Number of total cells)) x 100

The cells were transfected using serum free DMEM, containing 3 µL transfecting reagent (Mirus) and 1 µg plasmid DNA per 1 ml. For the co-transfections, 0.5 µg of each plasmid vector was used. The transfecting mixture was incubated for 20 min at room temperature, after which 50 µL of the mixture was added to the cells. Cells were incubated for 72 hr before the addition of substrates and test compounds (extracts and flavonoids). The expression vectors listed above were transfected into the cells. The plasmid, pCI-Neo, containing no DNA insert, was used as the negative control, and CYP11B1/pCMV was used as the positive control in the absence of test compounds.

5.2.4.2 Determination of steroid conversion assays in COS-1 cells

The influence of Rooibos and selected flavonoids was investigated on the catalytic activity of the P450 enzymes. Substrate conversion was assayed in the absence and presence of Rooibos extracts as well as flavonoid compounds. COS-1 cells that were transfected with the appropriate plasmids and PROG (1 µM) and PREG (3 µM) were used as substrate for CYP17A1, while PROG (1 µM) was used as substrate for CYP21A2. DOC (1 µM) and deoxycortisol (1 µM) were used as substrate for CYP11B1, while DOC (1 µM) was used as substrate for CYP11B2. CYP11B1 and CYP11B2 were co-transfected with ADX. Substrates were added to culture media with or without Rooibos extracts (4.3 mg/mL) or flavonoids (10 µM).

After 72 hr of incubation with transfection media, the media was replaced with culture media containing substrates in the presence and absence of extracts and test compounds. The cells were incubated and aliquots were removed for steroid extraction at specific time intervals, as indicated in the respective figure legends.

5.2.4.3 Culturing and maintenance of H295R cells

Although H295R cells express the full complement of human adrenocortical enzymes, the cell line is insensitive to ACTH stimulation (Xing et al. 2011). ACTH stimulates steroidogenesis in the adrenal glands, as mentioned in section 3.7.1 of Chapter 3. In addition to its role in steroidogenesis, ACTH is known to increase the expression of steroidogenesis enzymes in the adrenal cortex (Engeland et al. 1997). Forskolin, a diterpene, was used in the assays described below since the compound stimulates the adenyl cyclase (cAMP) pathways in adrenal cells (Seamon et al. 1981), which mimic the action of ACTH.

Frozen H295R cells (1 ml) were thawed and re-suspended by sieving them with a sterile cell sieve and they were subsequently grown in 10 ml sterile growth media (DMEM/F12, which contained 0.15 % $NaHCO_3$/L, 10 % cosmic calf serum, 1 % penicillin streptomycin and 0.05 % gentamycin) in a 100 mm tissue culture dish at 37 °C, 5 % CO_2, 90 %. The media was replaced with fresh media after every 24 hr. Detaching confluent cells from the tissue culture dish also involved 0.5 mL trypsin-EDTA, as described in section 5.2.4.1. For investigative purposes, confluent cells were replated into 12-well CellBind™ (Corning) dishes at 4×10^5 cells/ml/well after centrifugation through a cell strainer (40 µm) to remove cell aggregates. Cells were incubated for 48 hr in growth medium, which were then followed by a 12 h incubation process with growth medium containing 0.1 % cosmic calf serum, after which treatment of the cells commenced.

5.2.4.4 Determination of steroid production in H295R cells

The influence of Rooibos (1 mg/mL) extracts on steroid hormone production in H295R was assayed in the absence (basal conditions) and presence of forskolin (stimulated conditions). Basal conditions were assayed in the presence or absence of Rooibos extracts (final concentration of 1 mg/mL) for 48 hr. Stimulated conditions were assayed for 48 hr in the presence and absence of Rooibos extracts (final concentration, 10 µM), with the addition of

forskolin (final concentration of 10 µM). Aliquots of 500 µl were removed from each well, D4-cortisol (15 ng) was added as internal standard, after which the steroids were extracted and analysed as described in section 5.2.6.

5.2.5 Cell viability assay in the presence of Rooibos extracts

The effect of fermented and unfermented Rooibos extracts on cell viability was assayed to determine whether experimental concentrations affected the cells adversely. Confluent cells were replated into 12 well plates at 1×10^5 and incubated for 12 hr, after which the cells were incubated for 12 hr with culture medium containing 4.3 mg/mL Rooibos extract. Viability was assayed using the trypan blue dye exclusion method. Cells were harvested using 0.5 mL of trypsin. The cells were centrifuged at 500 ×g for 5 min at room temperature. Trypsin was aspirated off and the cells were resuspended in 0.5 mL of phosphate buffered saline. Viable cells were determined using trypan blue, as described in section 5.2.4.1.

Protein determination was carried out on the remaining cell suspension, as described in section 4.2.5 of Chapter 4, using the Pierce BCA Protein Assay Kit (Rockford, IL, USA) according to the instructions of the manufacturer. The cells were sonicated and a dilution series was prepared (10 X, 5 X and 2 X) for the determination.

Rooibos has been shown to have no effect on the viability of H295R cells (Schloms et al. 2012) and viability therefore was not assessed in this study.

5.2.6 Extraction and determination of substrate and product steroids

Steroids were extracted and quantified according to the method reported by Schloms et al. (2012). The media (500 µL) removed from the culture medium (see section 5.2.4) was transferred to centrifuge tubes containing 5 mL of dichloromethane, shaken for 20 min and centrifuged at 1200 ×g for 5 min. The media was removed and the dichloromethane was dried off under nitrogen gas at 40 °C. Dried steroids were resuspended in 150 µL methanol and transferred to glass vials for analysis by ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS). Steroids were separated using a Waters UPLC BEH and a Kinetix PFP column using solvent A (1 % formic acid) and solvent B (methanol:acetonitrile:isopropanol, 49 %:49 %:1 %). The solvents improve the chromatographic peak shape and provide a source of protons. Steroids were separated using a

linear gradient from 85 % A to 80 % B in 3.5 min, followed by a linear gradient from 80 % B to 100 % B in 0.1 min and an isocratic elution with solvent B for 1 min. Metabolites in a 5 ul sample volume were separated at a flow rate of 0.4 ml/min in 5 min.

The percentage inhibition of substrate conversion was calculated as follows:

$$\% \text{ Inhibition} = 100 - ((X/S) \times 100) \qquad \text{(Eq. 5.1)}$$

where X = % conversion of steroid in the presence of inhibitor, and S = % of steroid in the absence of inhibitor.

5.2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, California, USA) and calculated by a one-way ANOVA. All experiments were carried out in triplicate and the results are shown as means ± SEM. Values of $P < 0.05$ were considered statistically significant. Statistics are indicated in the respective figure legends.

5.3. Results

5.3.1 Analyses of Rooibos extracts

Extracts of unfermented and fermented Rooibos were analysed to determine the levels of the major flavonoid compounds. The data shown in Table 5.1 depicts the flavonoids (ug) extracted from one gram of Rooibos plant material. The levels of both dihydrochalcones were higher in the unfermented Rooibos than in the fermented Rooibos (aspalathin 32-fold and nothofagin 22.3-fold), while the aspalathin levels in unfermented Rooibos were 6.1-fold higher than nothofagin. Although the flavone levels were similar in the fermented and unfermented Rooibos, orientin and isoorientin were ± 6-fold higher than isovitexin and vitexin. Analysis of the flavonol compounds showed that, while quercetin-3-*O*-rutinoside appeared to be a more stable compound, rutin was 6-fold lower in the fermented extract.

Table 5.1. Flavonoid compounds (µg) present in the fermented and unfermented Rooibos extracts. Flavonoids were extracted from 1 g dried Rooibos plant material. Results are expressed as the mean ± SEM.

Flavonoids	Unfermented extract	Fermented extract
	µg	µg
Aspalathin	16838.7 ± 58.8	525.9 ± 8.3
Nothofagin	2755.5 ± 21.7	123.3 ± 2.7
Rutin	466.2 ± 0.7	125.0 ± 2.4
Quercetin-3-*O*-rutinoside	835.9 ± 2.4	749.8 ± 11.1
Orientin	1717.8 ± 7.0	1602.6 ± 25.4
Isoorientin	2129.1 ± 4.7	1848.7 ± 28.7
Vitexin	322.8 ± 1.7	358.2 ± 4.4
Isovitexin	368.1 ± 1.9	363.5 ± 6.2

The levels of flavonoid compounds present in the unfermented and fermented Rooibos extracts used in the experimental assays in COS-1 and H295R cells were analysed and are shown in Table 5.2. The data clearly shows that extracts of unfermented Rooibos administered to the cells contained significantly higher levels of dihydrochalcones than flavones and flavanols. Cells assayed in the presence of fermented extracts were exposed to higher levels of isoorientin, orientin and quercetin-3-*O*-rutinoside. Lower concentrations were used in the H295R overall steroidogenesis assays because the enzyme concentration is not known.

Table 5.2. Flavonoid compounds (µg) present in the Rooibos extract administered to COS-1 cells and H295R cells. Results are expressed as the mean ± SEM.

Flavonoids µg administered to cells				
Flavonoids	Unfermented extracts		Fermented extracts	
	H295R cells	COS-1 cells	H295R cells	COS-1 cells
Aspalathin	458.3 ± 1.6	106.6 ± 0.4	3.3 ± 0.1	14.3 ± 0.2
Nothofagin	75.0 ± 0.6	17.4 ± 0.1	0.8 ± 0.0	3.4 ± 0.1
Rutin	12.7 ± 0.0	3.0 ± 0.0	0.8 ± 0.0	3.4 ± 0.1
Quercetin-3-*O*-rutinoside	22.7 ± 0.1	5.3 ± 0.0	4.7 ± 0.1	20.4 ± 0.3
Orientin	46.8 ± 0.2	10.9 ± 0.0	10.1 ± 0.2	43.6 ± 0.7
Isoorientin	57.9 ± 0.1	13.5 ± 0.0	11.7 ± 0.2	50.3 ± 0.8
Vitexin	8.8 ± 0.0	2.0 ± 0.0	2.3 ± 0.0	9.7 ± 0.1
Isovitexin	10.0 ± 0.1	2.3 ± 0.0	2.3 ± 0.0	9.9 ± 0.2

The COS-1 cells were exposed to 4.3mg Rooibos extract/mL and H295R cells to 1mg Rooibos extract/mL.

5.3.2. Analyses of plasmid DNA

Plasmid DNA constructs encoding P450 enzymes were purified by alkaline lysis followed by anion exchange chromatography, and analysed spectrophotometrically using an ND-100 Spectrophotometer (NanoDrop; Thermo Scientific, USA). Optical densities were recorded at 260 nm and 280 nm and the purity of the isolated plasmids was determined (Table 5.3).

Table 5.3. Spectrophotometric results of harvested plasmid constructs.

Plasmid	pCI-neo	pCD-ADX	CYP11B1/pCMV	CYP11B2/pCMV	CYP17A1/pCI-neo	CYP21A2/pCI-neo
OD260:280	1.79	1.80	1.82	1.84	1.87	1.85
Total yield (μg)	270	520	1530	1390	830	1220

Although the ratios indicated the expected degree of purity (1.8), genomic contamination may affect downstream experiments. A further analysis was carried out using agarose gel electrophoresis, which also enabled the determination of DNA integrity. Analyses of the bands on the agarose gel (see Figure 5.1) showed that CYP17A1/pCI-neo and pCD-ADX CYP11B2/pCMV electrophoresed as two fragments. The lower, darker fragments represent the supercoiled plasmid DNA, as these migrated faster, whilst the lighter DNA fragments represent the nicked plasmid vectors, which migrated slower. CYP21A2/pCI-neo and CYP11B1/pCMV were detected as supercoiled DNA fragments only.

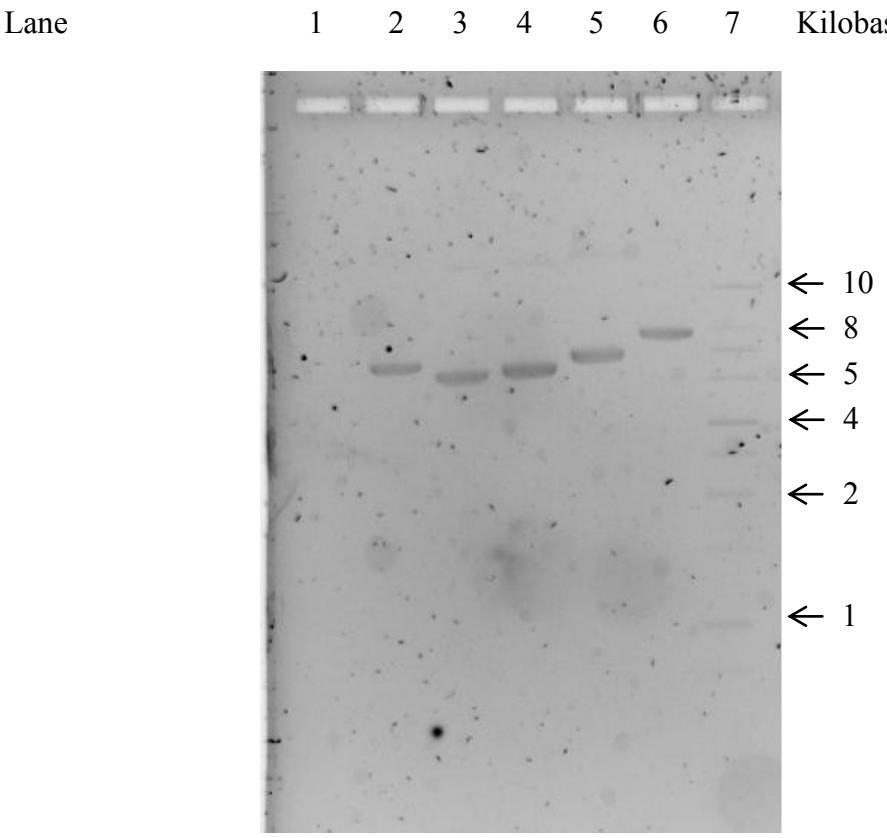


Figure 5.1. Agarose gel analysis of purified plasmid DNA. Lane 1: empty; Lane 2: CYP21/pCI-neo; lane 3: CYP17/pCI-neo; Lane 4: pCD-ADX; Lane 5: CYP11B2/pCMV; Lane 6; CYP11B2/pCMV Lane 7; molecular marker 1 kDa marker. DNA, 250 ng/well, was electrophoresed on a 1 % agarose gel, at a constant voltage of 70 V, and stained with ethidium bromide (1 ug/mL) and visualised on an ultraviolet light box

5.3.3 Cell viability

Prior to conducting enzymatic assays in the COS-1 cells, the cells were exposed to Rooibos extracts at the highest experimental concentrations (4.3 mg/mL) to determine the effect on

cell viability. The results indicated that, at this concentration, neither unfermented nor fermented Rooibos was toxic to the cells after a 12 hr incubation period. A standard protein determination was also carried out to determine whether cell growth was influenced and whether cell viability could be correlated to protein levels. There was no significant difference in cell viability and protein concentration ($P > 0.05$) when compared to the control cells, which were incubated without the addition of Rooibos extracts (Figure 5.2). The data show that Rooibos extracts did not affect protein content. Although a slight increase in cell viability and protein levels was observed, these were not statistically significant. As the data obtained reflected a similar trend to that obtained in previous studies, it was concluded that the viability of COS-1 cells was not affected by the concentrations used in experimental protocols.

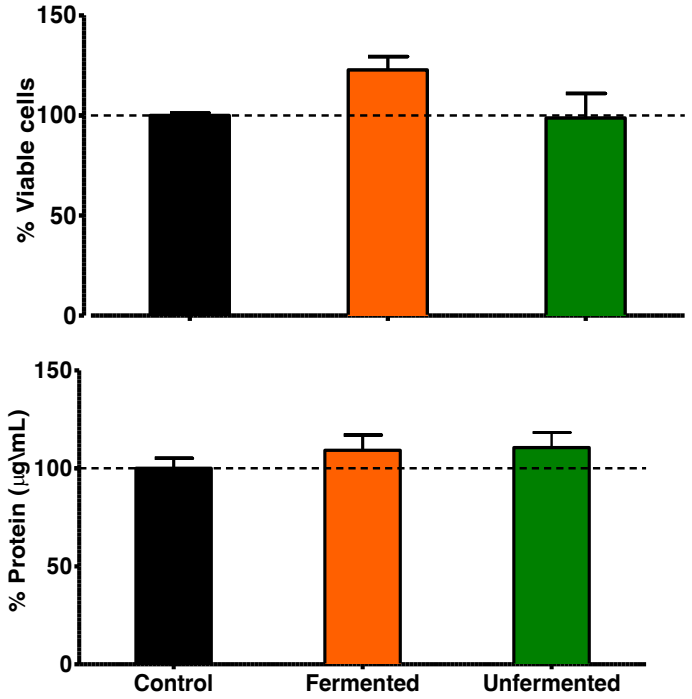


Figure 5.2. Cell viability and protein analyses of COS-1 cells incubated in the presence of 4.3 mg/mL unfermented and fermented Rooibos extracts. Results are a representative of one experiment with triplicate data points and were analysed using a one way ANOVA, followed by Dunnett's Multiple Comparison Test. Error bars represent ± SEM values.

5.3.4 Analyses of steroid conversion assays in COS-1 cells

The influence of Rooibos extracts (4.3 mg/mL) and selected flavonoids (10 µM) on the catalytic activities of the homologously expressed microsomal enzymes, CYP17A1 and

CYP21A2, and mitochondrial enzymes, CYP11B1 and CYP11B2, was investigated in COS-1 cells.

The conversion of PREG and PROG, both substrates of CYP17A1, was assayed in the presence and absence (control) of Rooibos and selected flavonoids after 8 hr (Figure 5.3). The results showed 13 % and 18 % inhibition of PREG conversion in the presence of unfermented and fermented Rooibos extracts respectively (Figure 5.3 A). PROG conversion by CYP17A1 was inhibited by 34 % and 50 % in the presence of unfermented and fermented Rooibos extracts respectively (Figure 5.3 B). Three flavonoid compounds were subsequently assayed, namely quercetin, rutin and aspalathin, with only the latter two influencing the substrate conversion by CYP17A1. PREG conversion by CYP17A1 was inhibited by 14 % and 36 % in the presence of rutin and aspalathin respectively (see Figure 5.3C). Rutin did not significantly affect the conversion of PROG; while it would appear that aspalathin stimulated the conversion of PROG (see Figure 5.3D). PREG was assayed at 3 μM instead of 1 μM, as previous studies conducted in our laboratory (results not published) showed that PREG assays at 3 μM give better product analyses than 1 μM PREG.

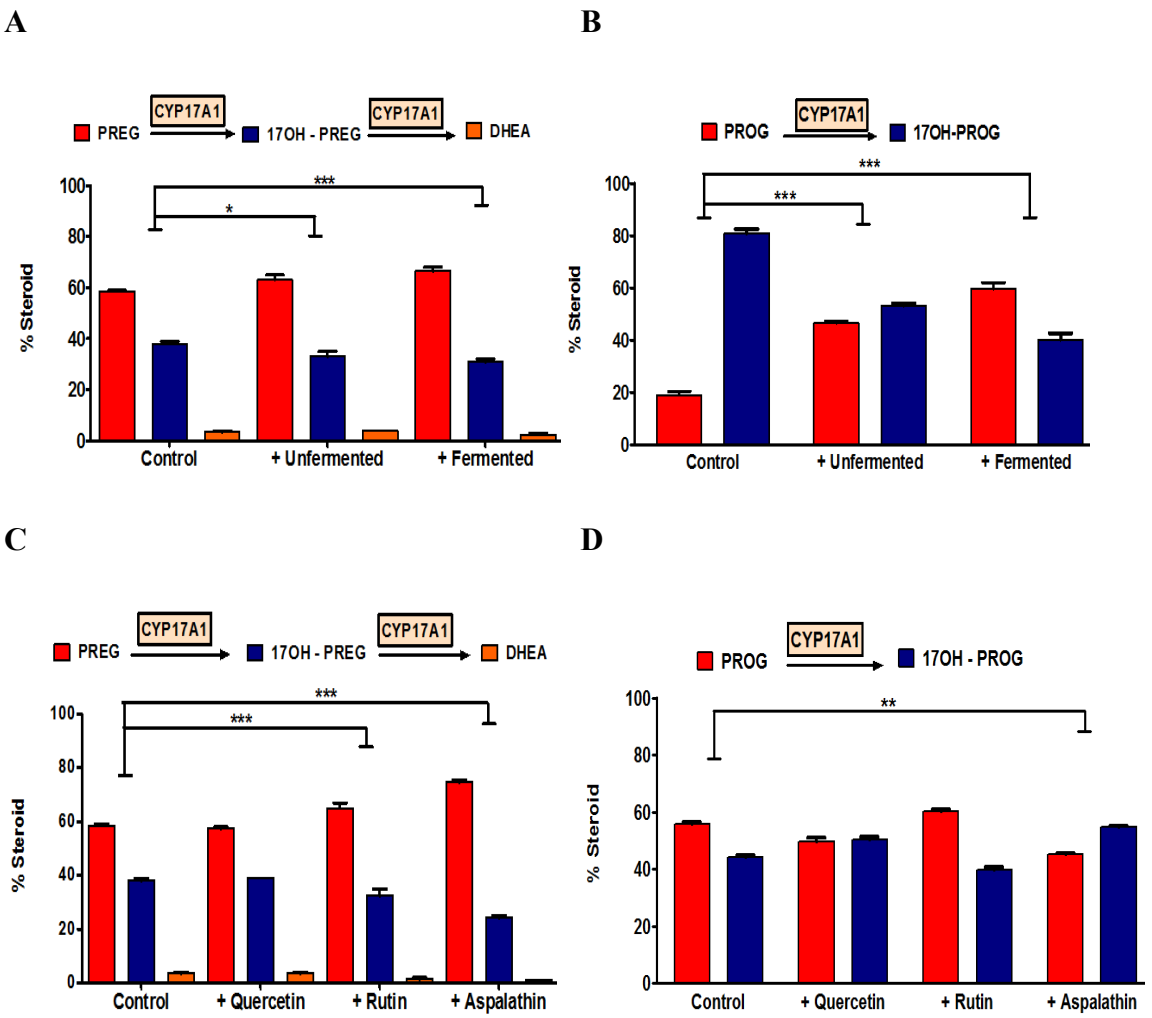


Figure 5.3. Influence of Rooibos extracts and selected flavonoids on PREG (3 µM) and PROG (1 µM) conversion in COS-1 cells by baboon CYP17A1. Percentage substrate and product with A) PREG and B) PROG in the presence of unfermented and fermented Rooibos extracts (4.3 mg/mL); C) PREG and D) PROG in the presence of quercetin (10 µM), rutin (10 µM) and aspalathin (10 µM), assayed after 8 hr. Individual steroids were compared by a Two-way ANOVA followed by Bonferroni's posttest. Results are presented as the mean error bars and represent ± SEM ($P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$ = ***; n = 3).

The conversion of PROG by CYP21A2 was assayed after 8 hr (Figure 5.4). The results showed significant inhibition of PROG conversion, which was 30 % and 90 % in the presence of unfermented and fermented Rooibos extracts respectively (Figure 5.4 A). Interestingly, of the flavonoids tested, quercetin exhibited the highest inhibition of PROG conversion, at 87 %, while 17 % and 24 % was obtained in the presence of rutin and aspalathin respectively.

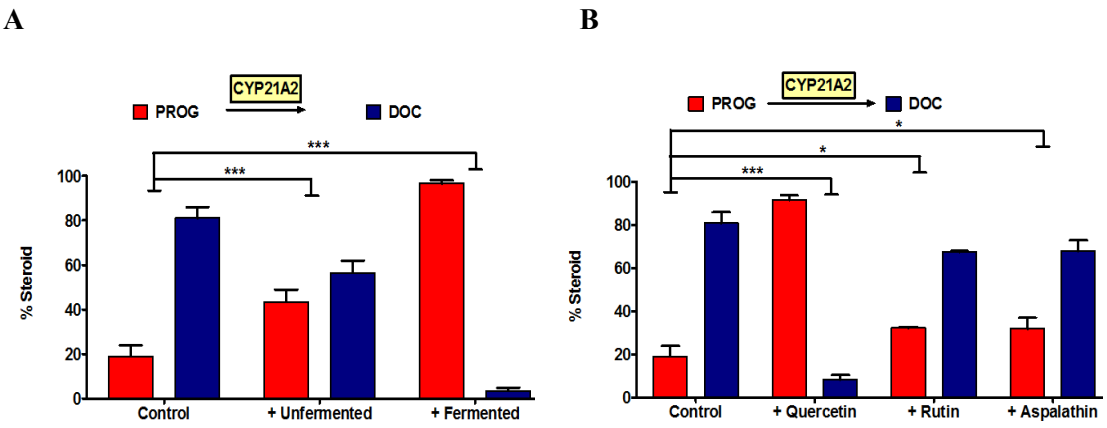


Figure 5.4. Influence of Rooibos extracts and selected flavonoids on PROG (1 µM) conversion in COS-1 cells by human CYP21A2. Percentage substrate and product with A) PROG in the presence of unfermented and fermented Rooibos extracts (4.3 mg/mL) and B) PROG in the presence of quercetin (10 µM), rutin (10 µM) and aspalathin (10 µM) assayed after 12 hr. Individual steroids were compared by a Two-way ANOVA followed by Bonferroni's posttest. Results are presented as the mean error bars and represent ± SEM ($P < 0.05$ = *, $P < 0.001$= ***; n = 3).

Both DOC and deoxycortisol are natural substrates for CYP11B1 and the influence of Rooibos on this enzyme was thus determined. In the presence of unfermented and fermented Rooibos, the inhibition of DOC conversion by the former was only 8 % with a greater degree of inhibition, 41 %, being obtained with the latter (Figure 5.5 A) after 8 hr. The inhibition of deoxycortisol conversion was greater than that of DOC, by 32 % by unfermented extract and 64 % by fermented extract after 6 hr (see Figure 5.5 B). While DOC conversion was not inhibited in the presence of any of the selected flavonoids (Figure 5.5 C), the inhibition of deoxycortisol conversion was also low with 10 % inhibition detected in the presence of rutin and aspalathin (see Figure 5.5 D).

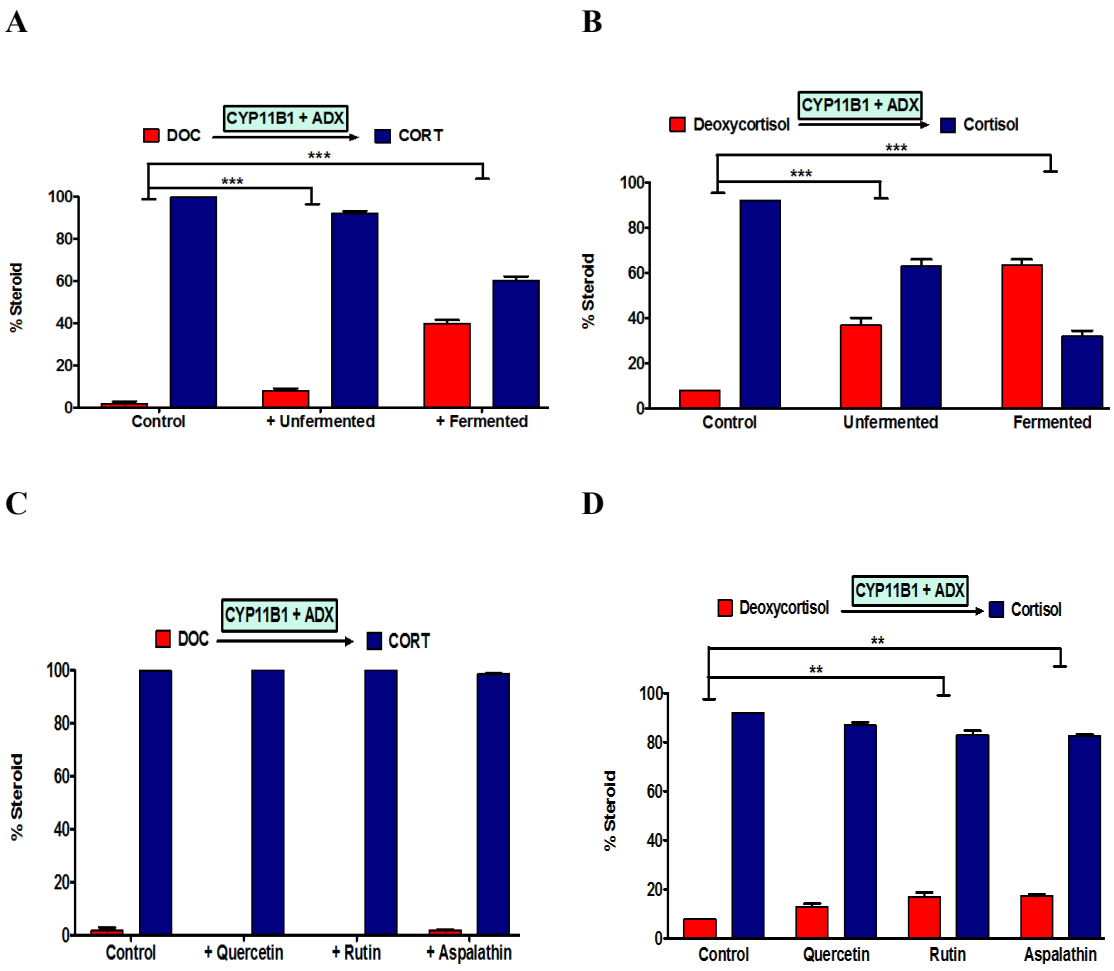


Figure 5.5. Influence of Rooibos extracts and selected flavonoids on DOC and deoxycortisol conversion in COS-1 cells by human CYP11B1 and ADX. Percentage substrate and product with A) DOC (1 µM) and B) deoxycortisol (1 µM) in the presence of unfermented and fermented Rooibos extracts (4.3 mg/mL); C) DOC (1 µM) and D) deoxycortisol (1 µM) in the presence of quercetin (10 µM), rutin (10 µM) and aspalathin (10 µM). DOC and deoxycortisol conversion was assayed after 8 and 6 hr, respectively. Individual steroids were compared by a Two-way ANOVA followed by the Bonferroni's posttest. Results are presented as the mean error bars and represent ± SEM ($P < 0.01$ = **; $n = 3$).

DOC conversion by CYP11B2 was assayed after 12 hr, as DOC is also a substrate for CYP11B2 in the mineralocorticoid pathway of adrenal steroidogeneis. CYP11B2 was inhibited at 26 % and 41 % in the presence of unfermented and fermented Rooibos extracts respectively (Figure 5.6 A). DOC conversion was inhibited significantly at 17 % in the presence of rutin while no inhibition was detected with quercetin or aspalathin (Figure 5.6 B).

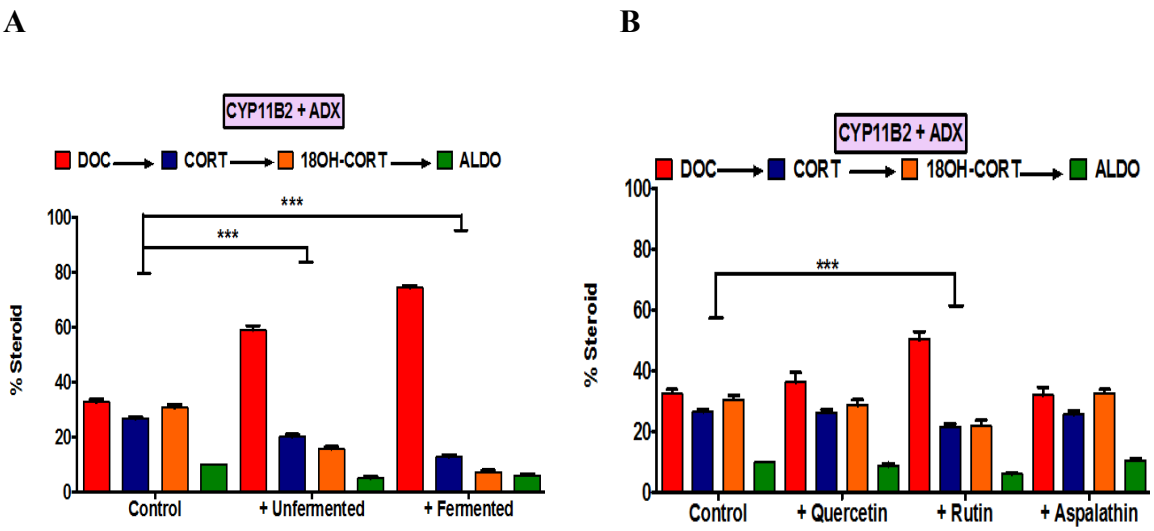


Figure 5.6. Influence of Rooibos extracts and selected flavonoids on DOC conversion in COS-1 cells by human CYP11B2 and ADX. Percentage substrate and product with A) DOC (1 µM) in the presence of unfermented and fermented extracts and B) DOC (1 µM) in the presence of quercetin (10 µM), rutin (10 µM) and aspalathin (10 µM), assayed after 12 hr. Individual steroids were compared by a Two-way ANOVA followed by the Bonferroni's posttest. Results are presented as the mean error bars and represent ±SEM ($P < 0.001$= ***; n = 3).

Empty plasmids (pCI-neo) that do not contain DNA insert were used as the negative control in the assays above. Cells were transfected with the vector, after which the same experimental protocol was followed. Steroid substrates were added and the media was analysed for products at appropriate times. Figure 5.7 shows the data collected for the control parameters in which the conversion of DOC (1 µM) by CYP11B1, expressed in COS-1 cells, was assayed as a positive control. The results, shown in Figure 5.7, indicate that the predominant hydroxylase activity of CYP11B1 in the positive control assay led to the production of CORT (steroid metabolite product), with negligible DOC being detected. No product was detected in the negative control assay, indicating that the activity detected was due to the expressed enzyme in the positive control.

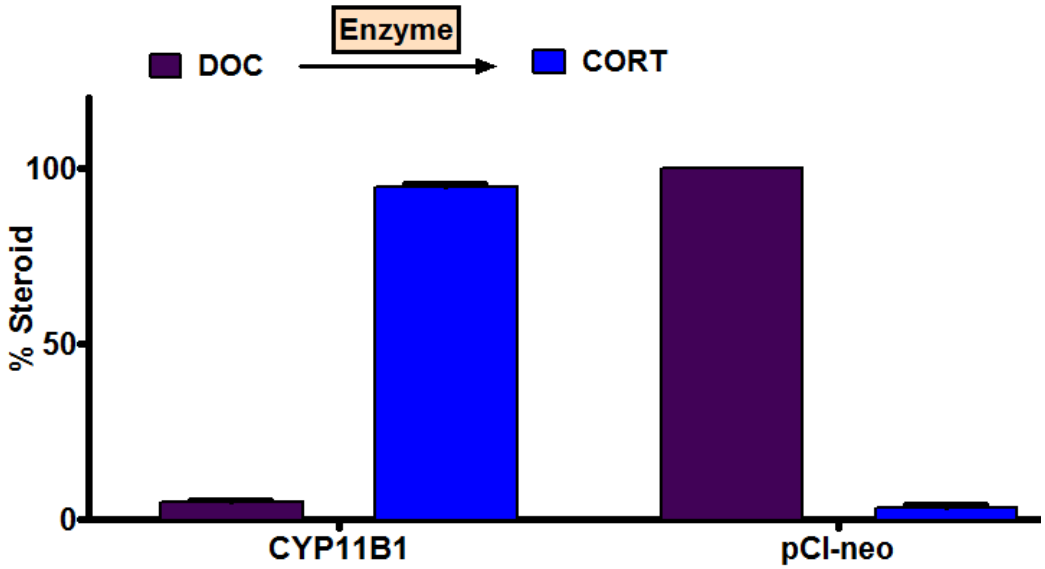


Figure 5.7. Positive and negative control assays in COS-1 cells transfected with CYP11B1/pCMV and pCI-neo plasmids respectively. Cells were incubated for 6 hr in the presence of DOC (1 µM). Results are of triplicate experiments with triplicate data points. Error bars represent ± SEM and n = 3.

5.3.5 Analyses of basal and forskolin stimulated steroid hormone production in H295R cells in the presence of Rooibos extracts.

The influence of unfermented and fermented Rooibos extracts on steroid levels was investigated in H295R cells that express all the steroidogenic enzymes. These cells produce the mineralocorticoids, glucocorticoids and adrenal androgens, thus making them an excellent model for studying steroidogenesis in the presence of Rooibos extracts under both basal and forskolin stimulated conditions.

The steroid hormone output under basal and stimulated conditions in the presence of Rooibos extracts, 1 mg/mL, was assayed after 48 hr. Under basal and forskolin stimulated conditions, unfermented Rooibos extract did not decrease the total steroid output significantly. In contrast, the fermented extract decreased the total steroid output under both basal and forskolin stimulated conditions by Two- and 3-fold respectively (Table 5.4). Under basal conditions, extracts of unfermented Rooibos had no significant influence on steroid metabolite levels other than testosterone. On the other hand, fermented Rooibos extracts increased PREG levels significantly, while significantly reducing basal steroid levels of CORT, 16OH-PROG, deoxycortisol, cortisol, testosterone and A4. The inhibitory effect of unfermented and fermented Rooibos extracts was more pronounced under stimulated

conditions, with both deoxycortisol and A4 levels being significantly decreased in the presence of Rooibos extracts. The decrease in all steroid metabolite levels was most significant in the presence of fermented Rooibos extract, resulting in an increase in PREG and PROG levels due to downstream inhibition of P450 enzymes.

Table 5.4. Steroid metabolites produced by H295R cells under basal and forskolin (10 μM) stimulated conditions in the presence of unfermented and fermented Rooibos extracts (1 mg/mL) after 48 hr.

Steroid	Basal	+ Unfermented Extract		+ Fermented Extract	
	nM ±SEM	nM ±SEM	Fold change	nM ±SEM	Fold change
PREG	99.4 ±13.7	92.5 ± 23.9		283.6 ± 64.4	2.9*↑
PROG	14.6 ± 1.3	17.2 ± 4.7		22.2 ± 6.3	
DOC	199.9 ± 27.9	335.0 ± 61.5		164.4 ± 29.6	
CORT	264.9 ± 10.4	364.3 ± 49.2		130.8 ± 30.9	2*↓
ALDO	2.7 ± 0.4	6.2 ± 3.0		4.2 ± 0.9	
17OH-PROG	44.3 ± 5.2	44.9 ± 7.8		48.4 ± 12.1	
16OH-PROG	92.3 ± 6.8	65.6 ± 10.3		27.0 ± 11.0	3.4**↓
Deoxycortisol	2686.5 ± 75.7	2102.1± 95.4		1245.2± 24.0	2.2**↓
Cortisol	1502.2±173.9	1252.9 ± 63.8		698.6 ± 132.2	2.2*↓
DHEA	104.8 ± 22.7	77.9 ± 19.3		77.6 ± 41.2	
DHEA-S	17.0 ± 3.8	6.6 ± 3.3		10.6 ± 0.5	
A4	698.6 ± 60.1	514.8 ± 68.9		237.3 ± 30.6	2.9***↓
11BOH-A4	173.8 ± 13.5	153.1 ± 25.9		110.5 ± 19.6	
Testosterone	32.7 ± 2.9	20.3 ± 3.9	1.6*↓	5.6 ± 1.6	5.8***↓
Total Steroids(nM)	6831.645	5814.867		3497.816	

Table 5.4. Continuation

Steroid	Forskolin	+ Unfermented Extract		+ Fermented Extract	
	nM ±SEM	nM ±SEM	Fold change	nM ±SEM	Fold change
PREG	690.4 ± 65.6	803.2 ±166.1		696.9 ±96.7	
PROG	31.1 ± 2.3	35.7 ± 4.0		38.5 ± 7.9	
DOC	838.1 ± 136.3	562.9 ± 88.8		260.0 ± 27.8	3.2**↓
CORT	2515.6± 311.9	2286.2± 313.9		497.3 ± 55.8	5***↓
ALDO	27.6 ± 5.0	35.0 ± 4.6		11.1 ± 0.8	
17OH-PROG	64.5 ± 10.9	30.8 ± 2.8		58.8 ± 16.4	
16OH-PROG	73.7 ± 6.7	43.6 ± 12.3		19.1 ± 5.4	3.9**↓
Deoxycortisol	2586.9 ± 496.6	1179.8 ±183.0	2.2*↓	785.0 ± 62.8	3.3**↓
Cortisol	4038.7 ±503.6	3619.0 ± 605.1		1124.7±93.3	3.6**↓
DHEA	339.6 ± 62.7	287.3 ± 23.7		260.3 ± 35.9	
DHEA-S	9.1 ± 0.0	3.3 ± 3.3		9.9 ± 0.8	
A4	642.1 ± 95.6	322.4 ± 58.6	2* ↓	145.4 ± 12.0	4.4***↓
11βOH-A4	207.4 ±10.4	199.6 ± 44.3		82.5 ± 7.4	2.5* ↓
Testosterone	15.5 ± 3.0	5.1 ± 0.8		1.2 ± 0.6	
Total Steroids(nM)	14444.3	11658.29		4658.784	

Fold changes, indicating only significant ↑ (increase) or ↓ (decrease) in steroid levels, were calculated from the changes in absolute values of the individual steroids compared to basal and forskolin stimulated values.

P values were calculated using One-way ANOVA followed by Dunnett's multiple comparison test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).Data is representative of a single experiment performed in triplicate. Steroids, (nmol/L) are expressed as the mean ± SEM (n = 3).

5.4. Discussion

The major polyphenols present in extracts of unfermented and fermented Rooibos were analysed in the current study. COS-1 cells were then incubated with these extracts to investigate their effects on P450 enzymes. Since COS-1 cells do not express P450 enzymes, expression vectors for specific P450 enzymes were transfected into these cells respectively. The influence of the extracts was also investigated on steroidogenesis in H295R cells that express all steroidogenic enzymes. It is widely reported that unfermented Rooibos contains higher levels of polyphenolic compounds than fermented Rooibos. It has also been shown that unfermented Rooibos contains significantly higher levels of dihydrochalcones, aspalathin and nothofagin than fermented Rooibos (Bramati et al. 2002; Joubert et al. 2008; Beelders et al. 2012). Analyses of the extracts used in this study also showed that the unfermented extracts contained higher levels of the polyphenolic compounds that were quantitated, with aspalathin and nothofagin being present at higher levels in the unfermented extracts (Table 5.2). Among the other major flavonoids analysed, all of which were found to be at similar levels in both the unfermented and fermented extracts, rutin was detected at significantly higher levels (3-fold) in the unfermented extracts. High levels of dihydrochalcones and rutin were also noted in the study conducted by Beelders et al. (2012). Remarkably, in both the unfermented and fermented extracts, isoorientin and orientin were present at higher levels than isovitexin and vitexin (± 5-fold). Similar findings were reported, where the mentioned flavones were detected at similar levels in unfermented and fermented extracts, with vitexin and isovitexin levels being lower than orientin and isoorientin (Beelders et al. 2012). The data in the current study correlate with polyphenol levels reported by Schloms et al. (2013), albeit at marginally lower levels. To date, 47 polyphenolic compounds have been identified in Rooibos (Joubert et al. 2008; Beelders et al. 2012), and not all phenolic compounds were analysed in the current study.

The influence of Rooibos extracts and selected flavonoids on the substrate conversion by heterologously expressed P450 enzymes was subsequently assayed. Included in the study was aspalathin, being the most abundant polyphenol compound in unfermented Rooibos. Rutin and quercetin represent flavonol compounds, with rutin, a diglycosylated derivative of the aglycone quercetin, also present at higher levels in unfermented Rooibos. Quercetin is only present in trace amounts in unfermented and fermented Rooibos and cannot be quantitated

accurately (Beelders et al. 2012). The flavonoid compound was included as it might be a representative of the absorbed form of rutin, having passed through the gut microbiome (Del Rio et al. 2013).

Previous studies conducted in our laboratory by Perold (2009) on the influence of Rooibos on steroid conversion in adrenal microsomes showed that unfermented and fermented Rooibos had similar inhibitory effects on the catalytic activity of CYP17A1 and CYP21A2. Both PREG and PROG conversion in adrenal microsomal preparations was significantly inhibited, at 90 % and 80 % respectively. Subsequent assays carried out in COS-1 cells transiently expressing these enzymes showed the inhibition of CYP21A2 to be higher than that of CYP17A1. In addition, while the inhibition of PROG conversion by CYP21A2 by unfermented and fermented Rooibos was similar (70 %), inhibition of CYP17A1 by unfermented extracts was ± 1.7-fold higher than that observed with fermented extracts (Perold 2009). In this study, both CYP17A1 substrates, namely PREG and PROG, were assayed. While the inhibition of PREG conversion by the two extracts was similar, the inhibition of PROG conversion was greater (1.5-fold) for fermented extract than unfermented extract. In addition, inhibition of PROG conversion by CYP21A2 in the presence of fermented extracts was also higher than the inhibition exhibited by the unfermented extract. Inhibition of the mitochondrial enzymes was also shown to be higher in the presence of fermented extracts, at 5-fold and 2-fold, in the case of CYP11B1 conversion of DOC and deoxycortisol respectively, and 2-fold in the case of CYP11B2 conversion of DOC. In a recent publication, Schloms et al. (2014) reported a 50 % and 55 % inhibition of PREG and PROG conversion by CYP17A1 and CYP21A2 respectively in the presence of unfermented extract, which is markedly higher than that found in the present study, namely 13 % and 30 % respectively. In the present study, PREG was assayed at a 3-fold higher concentration to facilitate product analyses, which also accounts for the high level of substrate remaining in the control assay after 8 hr. However, the inhibition of the conversion of PROG by CYP17A1 was 34 %, which was assayed at 1 μM in the presence of unfermented extracts. Since the assays conducted in COS-1 cells in the present study, as well as in the previous studies, were carried out using the same concentration of Rooibos extracts (dried extracted plant material/ml), the differences observed in the inhibitory effects may be due to differences in Rooibos plant material that resulted from natural variation, or the processing and storage of

experimental samples. Taken together it can nevertheless be concluded that both unfermented and fermented Rooibos influence the microsomal and mitochondrial P450 enzymes.

Aspalathin, which is a representative of the major polyphenolic compound in Rooibos, was subsequently assayed, as were rutin and quercetin. At 10 µM, aspalathin generally inhibited PREG and PROG conversion by CYP17A1 and CYP21A2 respectively, while exhibiting no inhibitory effect on CYP11B1 and CYP11B2. The inhibition of PREG conversion (36 %) was comparable to the inhibition (35 %) previously reported by Schloms et al. (2014). Interestingly, the data shows that aspalathin appears to stimulate the conversion of PROG to 17OH-PROG by CYP17A1. In the previous study, which showed inhibition in this case, PROG conversion was assayed for only 4 hr. It is also important to note that the unfermented and fermented extracts used in the assays contained different levels of aspalathin, at 1 mM and 31 µM, and rutin, at 20 µM and 5 µM. Rutin was previously shown to inhibit both the activity of CYP17A1 towards PREG, as well as the conversion of deoxycortisol by CYP11B1. Data indicated that the inhibition of CYP11B1 was substrate specific, as greater inhibition was detected with deoxycortisol than with DOC as substrate (Schloms & Swart 2014). In the present study, however, rutin was shown to exhibit a greater inhibitory effect towards CYP11B2, which was not investigated previously. Previous studies conducted in our laboratory showed rutin also inhibited the conversion of PROG by CYP21A2 at 3 µM and 10 µM (Perold 2009). It would seem from the data obtained in the present study that the flavonoids assayed had the greatest effect on CYP21A2 activity. The conversion of PROG was inhibited significantly (17 %); however, at the same concentration, the inhibition by quercetin was 87 %. It thus is possible that the rutinosyl moiety at C3 of rutin interferes with the flavonol binding to CYP21A2. The present study shows that the inhibition of the catalytic activity of the P450 enzymes obtained with the individual flavonoid compounds at 10 µM cannot be linked directly with the inhibition obtained by the unfermented and fermented rooibos extracts. These results indicate that there are other factors to consider when assaying the inhibitory activity of Rooibos extracts. The unfermented and fermented extracts contained higher levels of aspalathin, at 100-fold and 3-fold respectively, than the individual flavonoid, which was assayed at 10 µM. Rutin was present at 2-fold higher levels in the unfermented extracts and at lower levels in the fermented extracts. It is possible that other compounds present in the extract may interfere with the enzyme/substrate/compound interaction, or that

compounds may bind to each other when present in high concentrations and thus not be able to interfere with the formation of the enzyme/substrate complex.

Since Rooibos extracts influenced the steroidogenic P450 enzymes catalysing the biosynthesis of adrenal steroids in the COS-1 cells, the effect of the Rooibos extracts on steroid production was investigated in the H295R cells. As mentioned earlier in section 5.2, H295R cells retain the ability to secrete steroid hormones after stimulation with the ACTH, either Ang II or forskolin. Forskolin was used in this study, as H295R cells have a low response to ACTH due to the low expression of ACTH receptors (Mountjoy et al. 1994). The diterpene, forskolin, is an agonist of the protein kinase A pathway, thus mimicking ACTH effects (Rainey et al. 1994). Forskolin directly stimulates adenyl cyclase and has been used extensively to increase the cAMP involved in steroid biosynthesis (Seamon et al. 1981). Previous studies on the role of transport systems in cortisol release in H295R cells showed that forskolin increased mRNA expression for important enzymes in steroidogenesis. The mRNA expression of CYP11A1, 3βHSD, CYP17A1, CYP21A2 and CYP11B1 increased significantly (Asif 2004). Another study, conducted by Holland et al. (1993) on the influence of forskolin on H295R cells, showed an increase in steroid output after stimulation with 10 µM forskolin, and an increase of the mRNA of CYP11B2 in H295R cells (Holland et al. 1993).

Data analyses showed that forskolin increased the total steroid output by 2-fold with increased steroid metabolites in the mineralocorticoid, glucocorticoid and androgen pathways. The data obtained showed that, although the steroid flux through the androgen pathway increased, the mineralocorticoid and glucocorticoid pathways showed a greater increase in metabolites, with the end products, ALDO and cortisol, increasing 10-fold and 2.7-fold respectively in the presence of forskolin. In a study conducted by Winther et al. (2013), they showed that there was a 2-fold increase in cortisol output when H295R cells were stimulated with forskolin, which corroborates the current study's data showing cortisol output being increased by 2.7-fold in the presence of forskolin.

Under both basal and forskolin stimulated conditions, fermented Rooibos decreased total steroid output ± 2- and ± 3-fold. There also was a significant increase in PREG levels under basal conditions in the presence of fermented Rooibos extract, possibly due to the inhibition

of CYP17A1 or 3βHSD. A4 levels were reduced in the presence of both Rooibos extracts, suggesting the inhibition of 3βHSD under basal and forskolin-stimulated conditions. Studies by Ohno et al. (2002) on the inhibitory effects of phytoestrogen flavonoids in cAMP stimulated H295R reported a significant decrease of 3βHSD activity in the presence of low concentrations of flavonoids (IC_{50}= 0.5- 2.7 μM). The steroidogenic enzyme 3βHSD is a key enzyme that plays a central role at the branch point in steroid biosynthesis in adrenals. Moreover, the studies showed competitive inhibition of PROG and DHEA binding to CYP21A2 and 3βHSD respectively by one of the phytoestrogens, diadzein, at a Ki value of 2.9 μM. In the study, Ohno et al. (2002) suggested that the C6 and C3' hydroxyl groups on ring A and ring B of the flavonoids respectively were responsible for the inhibition of the steroidogenic enzymes. The study showed that 4'-hydroxyflavone, 6 -hydroxyflavone and apigenin with hydroxyl groups at C4', C5 and C7 significantly inhibited cortisol biosynthesis (Ohno et al. 2002). In the present study, the moieties at the above mentioned positions on the flavones and isoflavones in Rooibos could be responsible for the decrease in cortisol biosynthesis and total steroids under basal and forskolin stimulated conditions. However, changes in the 16OH-PROG levels will indicate an effect on CYP17A1 within steroidogenesis in the H295R cells, as this metabolite is a dead-end product that is not metabolised further. Under basal conditions, 16OH-PROG levels decreased in the presence of both Rooibos extracts, with a significant reduction detected in the presence of fermented Rooibos extract (3.9-fold). A similar inhibitory effect was obtained under stimulated conditions.

Data obtained from COS-1 cells expressing CYP21A2 indicated that the activity of this P450 enzyme is most sensitive to Rooibos extracts, as has also been shown in previous studies (Perold 2009; Schloms & Swart 2014). Under basal conditions in the H295R cells, regarding the conversion of PROG to DOC as well as 17OH-PROG to deoxycortisol, it was only the latter that was significantly decreased ($P < 0.01$) by fermented Rooibos extracts. However, under stimulated conditions, both DOC and deoxycortisol levels were decreased significantly ($P < 0.01$), with unfermented Rooibos extracts decreasing only deoxycortisol levels significantly ($P < 0.01$). The decrease in the levels of DOC and deoxycortisol resulted in lower levels of precursor metabolites being available for CYP11B2 and CYP11B1 in the mineralocorticoid and glucocorticoid pathways. Nevertheless, cortisol levels were decreased significantly in the presence of fermented Rooibos only under basal and forskolin stimulated

conditions. In addition, 11βOH-A4 levels were reduced under basal and forskolin stimulated conditions in the presence of Rooibos extract, suggesting the inhibition of CYP11B1, which reflects the data obtained in the COS-1 cells expressing CYP11B1.

The data obtained in this study shows that the fermented Rooibos extracts exhibited a greater inhibitory effect on steroidogenesis in the H295R cells, as well as on the steroidogenic enzymes expressed in COS-1 cells. Although the data obtained from the COS-1 cells is reflected in the inhibition of steroid metabolites in the adrenal cell model used in this study, further experiments and analyses will have to be conducted to confirm the differences observed in the data obtained using fermented and unfermented extracts.

5.5. Conclusion

From these observations it can be concluded that Rooibos, both unfermented and fermented, influences the catalytic activity of steroidogenic enzymes and thus modulates steroidogenesis. These findings provide scientific evidence that rooibos herbal infusions may influence the endocrine system via adrenal steroidogenesis and may have health benefits in the management of endocrine related diseases.

CHAPTER 6

Conclusion

The argument made for medicinal and health benefits of Rooibos infusions are based partly on scientific evidence and partly on anecdotal evidence. There is much epidemiological evidence that diets rich in the bioactive compounds found in plants can reduce the incidence of non-communicable endocrine related diseases, such as cardiovascular diseases, diabetes, cancer and stroke. To date, there is limited information on the effects of Rooibos consumption on cytochrome P450 enzymes. As a result, it is imperative to investigate the possible effects of Rooibos, and of its phenolic secondary metabolites, on the endocrine system, specifically with regard to the P450 enzymes. In this study, the focus was thus on the influence of Rooibos and major Rooibos flavonoid compounds on some of the key P450 enzymes involved in adrenal steroid hormone biosynthesis. Adrenal hormones play a crucial role in some of the endocrine related diseases, such as stress, anxiety, diabetes and hypertension.

Since adrenal steroid biosynthesis may be influenced by Rooibos interfering with or influencing the binding of natural substrates to P450 enzymes, this aspect was investigated in ovine microsomal and mitochondrial preparations. It was shown that unfermented and fermented Rooibos extracts, at similar concentrations, interfered with DOC binding to the mitochondrial P450 enzymes (CYP11B1 and CYP11B2) to a greater degree than PROG binding to the microsomal P450 enzymes (CYP17A1 and CYP21A2). In addition, substrate binding to CYP17A1 and CYP21A2 was inhibited to a greater extent with unfermented Rooibos extract than with fermented Rooibos extract. The influence of selected flavonoids, namely aspalathin, quercetin and rutin, was subsequently assayed. Microsomal P450 enzymes were inhibited by rutin to a greater extent than quercetin, although quercetin showed greater substrate binding inhibition of mitochondrial enzymes. These investigations also showed that aspalathin did not affect substrate binding in a dose dependent manner. In this investigation, rutin exhibited greater inhibitory capabilities than quercetin at half the concentrations used for quercetin. The inhibition by rutin and quercetin may be attributed to the structural similarities they have with the natural substrates (PREG and PROG), hence their name phytoestrogen. The differences detected in the interference of substrate binding to the P450

enzymes by the Rooibos extracts may be due to the degradation of the polyphenols as a result of their instability during the fermentation process. In addition to the structural similarities with natural substrates, it is also possible that these flavonoids could have interfered with the electron transport system discussed in Chapter 3, as it is widely reported that polyphenolic compound can act as electron scavengers.

Taken together, the substrate binding study showed that rooibos extracts, as well as the selected flavonoids, interfered with substrate binding to adrenal P450 enzymes. This data, however, does not necessarily imply that the catalytic activity of P450 enzymes is influenced; hence substrate conversion assays in COS-1 cells and H295R cells were carried out to complete the second aim.

The influence of unfermented and fermented Rooibos extracts and selected flavonoids on the catalytic activity of P450 enzymes expressed in the COS-1 cells showed that fermented extracts inhibited the catalytic activity of both microsomal and mitochondrial P450 enzymes to a greater degree than unfermented extracts. It is interesting to note that, while none of the selected flavonoids influenced CYP11B1's catalytic activity, the microsomal enzymes were inhibited by these compounds. The inhibition of the catalytic activity of CYP21A2 by quercetin was greater than by rutin and aspalathin, and while rutin had a greater inhibitory effect on CYP17A2 than quercetin and aspalathin, the latter inhibited the catalytic activity of CYP17A1 only. Further experiments need to be conducted to establish the mechanisms by which Rooibos flavonoid compounds inhibit P450 enzymes. Although the Rooibos compounds assayed were shown to interfere with the binding of natural substrates to specific enzymes, it has not been established whether these compounds bind to the active site, or whether they bind to another domain on the protein molecule, possibly leading to a conformational change hampering substrate binding. An investigation of competitive, uncompetitive and/or non-competitive inhibition by flavonoid polyphenols may shed light on the differences observed in the binding and conversion assays that were conducted in this study.

In the view of the fact that Rooibos extracts had an effect on substrate binding to P450 enzymes and substrate conversion by P450 enzymes, the effect of unfermented and fermented Rooibos extracts on adrenal steroidogenesis was investigated in an adrenal cell model, the

H295R cell line. Although the data shows that, in the presence of Rooibos extracts, total steroid hormone production decreased in the H295R cells, it is clear that, under basal conditions, total steroid levels were decreased to a lesser degree, with a more pronounced decrease in total steroid production being detected in the presence of both unfermented and fermented extracts under forskolin stimulated conditions. The analyses also showed that a decrease in the levels of the products of reactions catalysed by CYP17A1 (specifically 16-OHPROG), CYP21A2 (DOC, deoxycortisol), CYP11B1 (cortisol) and CYP11B2 (CORT) will result in a decrease in the end products of adrenal steroidogenesis. The data obtained also reflect the inhibition detected in the COS-1 cells assays, in which substrate conversions catalysed by CYP17A1, CYP21A2, CYP11B1 and CYP11B2 inhibition were greater in the presence of fermented extracts than in the presence of unfermented extracts. It can be concluded that unfermented and fermented Rooibos affect adrenal steroidogenesis in H295R cells by decreasing the amount of total steroids produced. It is clear that the decrease observed in precursor levels consequently leads to the decrease in aldosterone and cortisol levels, most prominent under forskolin stimulated conditions, suggesting that Rooibos may aid in the treatment of hypertension and stress related diseases.

It generally can be concluded that the inhibition detected in the substrate binding assays and in the substrate conversion assays, as well as the effect of Rooibos extracts on steroidogenesis in H295R cells, cannot be attributed specifically to the flavonoids analysed, as 46 flavonoid compounds have been identified in Rooibos to date, some of which are present at higher levels than those analysed in this study. It is also possible that, of all the flavonoids present in Rooibos, some may inhibit steroidogenic enzymes, while others may not, and while some may have no effect at all, polyphenols may also stimulate these enzymes.

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