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

by

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Declaration

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Abstract

This study:

1. Describes the preparation of unfermented and fermented rooibos methanol and aqueous extracts.

2. Investigates the influence of unfermented and fermented rooibos methanol and aqueous extracts on the binding of natural steroid substrates to ovine adrenal microsomal cytochrome P450 enzymes, demonstrating that the binding of natural steroids is inhibited in the presence of rooibos extracts.

3. Describes an assay demonstrating the inhibitory effect of rooibos extracts on the catalytic activity of cytochrome 17α-hydroxylase (CYP17) and cytochrome 21-hydroxylase (CYP21) in ovine adrenal microsomes.

4. Investigates the influence of unfermented and fermented rooibos methanol extracts on the catalytic activity of individual cytochrome P450 enzymes – CYP17 and baboon CYP21, that are expressed in COS1 cells.

5. Demonstrates that fractions of the unfermented rooibos methanol extract inhibits the binding of natural steroid substrate to microsomal cytochrome P450 enzymes as well as the catalytic activity of baboon CYP21 expressed in COS1 cells.

6. Investigates the inhibitory influence of individual rooibos flavonoids on the catalytic activity of baboon CYP21 expressed in COS1 cells.

Opsomming

Hierdie studie beskryf:

1. Die voorbereiding van gefermenteerde en ongefermenteerde rooibos metanol- en waterekstrakte en die fraksionering van die ongefermenteerde rooibos metanolekstrak.

2. ’n Ondersoek na die inhibisie van die binding van natuurlike steroïdsubstrate, progesteroon en 17-hidroksi-progesteroon, aan sitochroom P450 ensieme in skaap bynier mikrosome in die teenwoordigheid van gefermenteerde en ongefermenteerde rooibos metanol- en waterekstrakte.

3. Die inhiberende invloed van die gefermenteerde en ongefermenteerde rooibos ekstrakte op die katalitiese aktiwiteit van sitochrome P450 17α-hidroksilase (CYP17) en sitochrome P450 21-hidroksilase (CYP21) in skaapbyniermikrosome.

4. Die inhibisie van CYP17 en CYP21 wat in COS1 selle uitgedruk is in die teenwoordigheid van die gefermenteerde en ongefermenteerde rooibos metanolekstrakte.

5. Die inhiberende effek van geïsoleerde rooibos fraksies op progesteroon binding aan mikrosomale sitochroom P450 ensieme en op die CYP21 gekataliseerde omsetting van progesteroon in COS1 selle.

6. Die inhibisie van flavonoïed verbindings, geïdentifiseer in rooibos, op die katalitiese aktiwiteit van CYP21 uitgedruk in COS1 selle.

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Abbreviations

17OH-PREG	17α-hydroxypregnenolone
17OH-PROG	17α-hydroxyprogesterone
17βHSD	17β-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
5´-DI	iodothyronine 5′-deiodinase
AAF	acetylaminofluorene
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
ACTH	adrenocorticotropic hormone
ADX	adrenodoxin
ADXR	adrenodocin reductase
AFB_1	aflatoxin B1
B(a)P	benzo[a]pyrene
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
B_{max}	maximum substrate binding capacity of enzyme
BSA	bovine serum albumin
cAMP	adenosine 3'5'-cyclic monophosphate
CCl_4	carbon tetrachloride
CHP	cumolhydroperoxide
CNS	central nervous system
COS1 cells	transformed Africa green monkey kidney tumor cells
COX-2	cyclooxygenase-2
CRE	cAMP-responsive element
CRH	corticotrophin releasing hormone
CYP101	cytochrome P450 cam
CYP11A	cytochrome P450 side chain cleavage
CYP11B1	cytochrome 11β-hydroxylase

CYP11B2	aldosterone synthase
CYP17	cytochrome 17α-hydroxylase/17,20 lyase
CYP19	aromatase
CYP1A2	cytochrome P450 dependent monooxygenase 1A2
CYP21	cytochrome 21-hydroxylase
DHEA	dehydroepiandrosterone
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagles Medium
DOC	11-deoxycorticosterone
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EDCs	endocrine-disrupting chemicals
FMN	flavin mononucleotide
FSH	follicle stimulating hormone
GR	glucocorticoid receptor
GSH	reduced gluthathione
GSSH	oxidized gluthathione
GST-α	glutathione S-transferase alpha
HDL	high density lipoproteins
HPA axis	hypothalamic-pituatary-adrenal axis
HPLC	high performance liquid chromatography
HPLC	high performance liquid chromatography
IL	interleukin
Kd	equilibrium dissociation constant of the ligand
K_i	binding inhibition constant
K'_i	apparent binding inhibition constant
K_m	Michaelis constant
K_s	binding constant
K'_s	apparent binding constant
LCMS	liquid chromatography mass spectrometry
LH	luteinizing hormone

MDA	malondialdehyde
MMC	mitomycin C
MNRET	micronucleated reticulocytes
MR	mineralocorticoid receptor
MSH	melanocyte-stimulating hormone
NAD(H)	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
OVA	ovalbumin
PEG	polyethylene glycol
PEPCK	phosphoenolpyruvate kinase
PhIP	2-amino-1-methyl-6- phenylimidazo [4,5-b]pyridine
PKA	protein kinase A
POMC	pro-opiomelanocortin
PREG	pregnenolone
PROG	progesterone
PVN	paraventricular nucleus
ROS	reactive oxygen species
SEM	standard error of the mean
SRBC	sheep red blood cell
StAR	steroidgenic acute regulatory protein
SULT1C1	sulfotransferase 1C1
T3	3,5,3′-triiodothyronine
T4	L-thyroxine
TAA	total antioxidant activity
TBARS	thiobarbituric acid reactive substances
Th1 and Th2	helper T lymphocyte type 1 and type 2
TNF	tumor necrosis factor
TPA	12-O-tetra-decanoylphorbol-13-acetate

Tris	tris(hydroxymethyl)aminomethane
UDP-GT	UDP-glucuronosyl transferase
V_{max}	maximum reaction rate

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Chapter 1

Introduction

The African continent is rich in medicinal plants and the numerous cultures and traditions integrally link their use of plants within their communities. In the search for new plant-based therapies and products to improve health and nutrition, a great deal can be learned from the traditional healers and indigenous people in the application of medicinal plants. African medicines need to be developed to lead to increased trade and economic benefits for Africans, together with scientific contributions, while still respecting African traditional medicine [Simon *et al*., 2007].

Rooibos (*Aspalathus linearis*), one of the plants that has been used as a traditional remedy is a plant indigenous to South Africa. The popularity of rooibos in the international market is escalating and this phenomenon can be ascribed to its health promoting properties. Although rooibos is also available in various guises such as creams, hair lotions, soaps and other skin care products in an ever increasing market, it is mainly consumed as a herbal tea. It is commercially available as a fermented product but is also appearing on the market in the unfermented form. Processing of rooibos is carried out in two different ways to produce two types of tea – fermented and unfermented rooibos. Unfermented rooibos is immediately dried after harvesting to prevent oxidation and is called green rooibos tea. During fermentation, the leaves of rooibos turn an orange red color due to the oxidation of compounds. Studies have confirmed its antioxidant, hepatoprotective and antimutagenic properties and consumption of large quantities of rooibos do not appear to have any negative effects on the human body [Joubert *et al*. 2004, 2005; Ulicna *et al*. 2003].

Rooibos tea has traditionally been used as a herbal remedy to treat various stress related ailments linked to the endrocrine system. Dysregulation of the stress response is associated with elevated glucocorticoid levels. The biosynthesis of glucocorticoids, together with mineralocorticoids and

androgens are catalysed by cytochrome P450 (P450) enzymes in the human adrenal gland. Inhibition or activation of these enzymes would have a major impact on the endocrine system by affecting the synthesis of glucocorticoids and mineralocorticoids. P450 enzymes are therefore crucial to normal endocrine function. Investigations were thus carried out to determine the influence of rooibos on the adrenal P450 enzymes to ascertain whether it may exhibit anti-stress properties via its influence on adrenal steroidogenesis.

In chapter 2 the history and processing, as well as the phytochemical composition and biological activity, of rooibos is discussed. Rooibos contains a wide variety of polyphenols. Aspalathin and nothofagin are the major flavonoids of rooibos tea and ~43% of the total antioxidant capacity of aqueous extracts of unfermented rooibos [Schulz *et al*., 2003; Von Gadow, 1997] can be attributed to aspalathin, with rooibos being the only known natural source of aspalathin reported to date [Joubert, 1996]. Unfermented rooibos is characterized by a higher level of polyphenol antioxidants and research has demonstrated that the levels of aspalathin in unfermented rooibos are much higher than its fermented counterpart [Bramati *et al*., 2003]. Research into the phytoestrogenic properties of rooibos has identified compounds in rooibos that possess mild estrogenic activity [Shimamura *et al*., 2006]. These compounds show structural homology with steroids and could therefore hamper their binding to receptor and carrier proteins and enzymes as well as their metabolism. Studies reporting the antimutagenic activity of rooibos suggest that the extracts may induce its effect by interfering with cytochrome P450-mediated metabolism of carcinogens that require metabolic activation [Marnewick *et al*., 2000]. It is thus possible that rooibos may interact with adrenal steroidogenic P450 enzymes.

Rooibos has been used for the treatment of anxiety, depression, nervous tension, atherosclerosis and diabetes even though these claims have not been scientifically verified. These factors are all associated with abnormal high cortisol levels, impacting negatively on the endocrine system. During stress, the hypothalamic-pituitary-adrenal (HPA) axis of the endocrine system is activated and glucocorticoids, cortisol and corticosterone, are secreted. Although the function of the stress response is to maintain homeostatis, chronic activation of the stress system and exposure to elevated cortisol levels can have adverse and detrimental effects on the body even

leading to death in extreme cases. The response of the body to stress and the effects of a chronically activated HPA axis are discussed in chapter 3.

Cortisol is the major stress hormone that is synthesised in the adrenal cortex and the enzymes responsible for the biosynthesis of this hormone are the steroidogenic cytochromes P450. The P450 enzymes represent a diverse superfamily of hemoproteins found in all lineages of life. An overview of the P450 enzymes is presented in chapter 4 with specific focus on the adrenal steroidogenic P450 enzymes. The unique spectral properties of P450 enzymes are a valuable tool that allows for the analyses of the binding of substrate to these enzymes by spectrophotometry. These spectral characteristics can be used to investigate substrate affinity and the effect of enzyme inhibitors on the binding of substrate to the P450 enzymes. CYP17 and CYP21 are two P450 enzymes that play a key role in the adrenal steroidogenesis pathway and inhibition of these enzymes would significantly influence cortisol plasma levels [Arlt and Stewart, 2005].

In chapter 5 an investigation into the biological properties of rooibos is described. The influence of extracts from the plant on the adrenal steroidogenic P450 enzymes, CYP17 and CYP21 is presented. Spectral binding assays were performed to determine the effect of fermented and unfermented extracts on the binding of progesterone (PROG), a natural steroid substrate. The influence of rooibos extracts on the catalytic activity of the P450 enzymes was subsequently investigated by conducting metabolic assays in microsomal preparations containing both CYP17 and CYP21. CYP17 and CYP21 were also expressed in COS1 cells and the influence of rooibos and flavonoid compounds on the catalytic activity of the individual enzymes was investigated. The results are discussed in chapter 5.

A summary of the results obtained in this study and the conclusions which were drawn from the data are presented in chapter 6.

The aims of this study were:

- To prepare fermented and unfermented rooibos methanol and aqueous extracts

- To investigate the influence of rooibos on two key P450 enzymes in the steroidogenic pathway, CYP17 and CYP21, using spectral assays and metabolic conversion assays
- To fractionate extracts that exhibit a high degree of inhibition
- To identify bioactive compounds in rooibos that influence the steroidogenic P450 enzymes

Chapter 2

Aspalathus linearis (Rooibos)

2.1 Introduction

Rooibos (*Aspalathus linearis, Fabaceae*) is a leguminous shrub native to the mountainous areas of the northwestern Cape Province in South Africa [Erickson, 2003; Van Heerden *et al*., 2003]. The plant is unique to South Africa and it was, as is the case with most African plants, the local inhabitants of the Cederberg Mountains who first discovered that the shrub-like plant can be used as a tea, with an exceptional taste and aroma.

South Africans have long been aware of the health properties and versatility of rooibos. Today, these qualities are also being embraced by a rapidly growing number of loyal rooibos tea drinkers nationally and internationally, as evidenced by the increasing popularity of rooibos in countries as diverse as Germany, Japan, the Netherlands, England, Malaysia, South Korea, Poland, China, and the United States [Erickson, 2003].

It has long been known that secondary metabolites in plants possess biological activities [Robak *et al*., 1996]. Several traditional cultures remain, to date, dependent on plants for their food and medicinal needs, often considering both in the same context [Huffman, 2003]. Approximately 80% of the global population relies on indigenous or traditional medicines for their primary health needs, with most of this practice involving the use of plant extracts, often in aqueous solutions [Zhang, 2002]. The use of herbal preparations has prevailed for centuries and health care providers in Europe and Asia often prescribe herbal teas. Such practices are however, mostly based on folklore and schools of traditional medicine rather than on scientifically based research data. It appears that in most cases the bioactivity of these plants is derived from secondary metabolites, such as polyphenols [Huffman, 2003].

Tea (*Camellia sinensis*) has been the subject of a number of studies during the last decade, linking consumption to a reduced risk for cancer in humans. These studies have led to an increase in the popularity and credibility of tea as a health drink with chemopreventive properties. On the other hand, concerns regarding the detrimental effects of caffeine on health have increased the consumption of decaffeinated teas such as rooibos [Van der Merwe *et al*., 2006].

Rooibos tea infusions are reported to exhibit antioxidant activity which can be attributed to the presence of polyphenols. Plants rich in polyphenols are used by the food industry as antioxidants to enhance the apparent health promoting properties of food products. Processed foods are often enriched with polyphenols as a protective measure against oxidation, extending the shelf life as the formation of toxic products, like cholesterol oxides, are being prevented [Joubert *et al*., 2005]. These polyphenols, also known as flavonoids, are more abundant in unfermented than in fermented rooibos. Some of these phenolic compounds identified in rooibos show structural homology with steroid hormones and have been shown to exhibit phytoestrogenic activity. Although phytoestrogens are readily metabolized, these compounds can have significant effects on the endocrine system [Mesiano *et al*., 1999].

Rooibos tea is a safe beverage for infants, children and pregnant women and has not been shown to have adverse physiological effects [Erickson, 2003]. Rooibos is reported to have various therapeutical properties, such as calming digestive disorders and various stomach problems as well as alleviating allergies [Bramati *et al*., 2003]. In South Africa, it is also used as a treatment of colic in babies [Erickson, 2003]. Rooibos tea also exhibits anti-depressive properties which counteract nervous tension and insomnia [Otto *et al*., 2003, Bramati *et al*., 2003]. Anxiety, depression and nervous tension are all factors associated with high cortisol plasma levels that may result from a dysregulation of the stress response, impacting negatively on normal endocrine functions [Tsigosa and Chrousos, 2002]. It is possible that the polyphenols in rooibos tea could influence the endocrine system by interacting with the enzymes involved in the pathway of cortisol biosynthesis. The phenolic composition and the bioactivity of compounds in rooibos are reviewed in this chapter.

2.2 Origin and nomenclature

Rooibos has been used by the indigenous Khoi-Khoi tribe of the Cederberg region since 1772 (figure 2.1) [Morton, 1987]. They discovered the medicinal value of this tea and harvested the plant with axes, bruised it with hammers and left it to ferment in heaps, before drying it in the sun [Wilson, 2005]. Technically infusions made from the leaves of the *Camellia sinensis* plant are referred to as tea — the correct term for infusions made from herbs such as rooibos is tisane. However, tea is the term commonly used for herbal infusions and will therefore be used as such. Tea is processed in three different ways, producing different types of tea – unfermented green and white tea, partially fermented oolong tea, and fermented black tea [Erickson, 2003; Pilar *et al*., 2008].

In 1904, Benjamin Ginsberg, a descendant from a family who had been in the tea industry in Europe for centuries, became interested in rooibos and realised its marketing potential. He started buying tea from the local people in the Cederberg Mountain region and resold it on the South African market. This was the beginning of a profitable new industry [Wilson, 2005].

It was in the late 1920's when rooibos became a cultivated crop and it has been grown commercially since World War II [Erickson, 2003]. Rooibos is becoming increasingly popular, with the total production of rooibos, including unfermented rooibos, estimated at being in the excess of 14 000 tons for 2007. The international demand for rooibos tea has grown from 750 tons in 1993 to 7200 tons of rooibos in 2007. The major international market for rooibos is Germany (53 %), followed by the Netherlands (11 %), UK (7 %), Japan (6 %) and the USA (5 %) [Joubert *et al*., 2008].

Figure 2.1. Rooibos growing in the Cederberg region of South Africa [www.rooibosltd.co.za].

2.3 Processing

Rooibos made a successful transition from a wild to a cultivated crop and is one of the few economically important fynbos plants. Rooibos seeds are sown from February to March and seedlings are replanted during July and August in the Southern hemisphere winter period. Only the top half of the plant is cut, with about 30 cm left above the soil (figure 2.2). It is vital to make sure that healthy leaves remain on the plant after harvesting or else the plant will not survive. In addition during the second year of harvesting, the plant should not be cut below the height harvested the previous year. It is best to harvest slightly higher each year for new growth can come from the previous season's wood. Rooibos is harvested once a year between December and April [www.asnapp.org.za].

As previously mentioned rooibos was harvested with axes by the Khoi-Khoi tribe —bruised, fermented and dried in the sun [Wilson, 2005]. Today rooibos is still processed in a similar

manner, although the process has been mechanized. Processing is carried out in two different ways to produce the two types of tea — after harvesting, the leaves and stems are either bruised and fermented to produce the traditional fermented rooibos or immediately dried to prevent oxidation, producing unfermented rooibos. Before packaging, the dry product is sterilized by steam pasteurization [Standley *et al*., 2001].

Rooibos tea is referred to as fermented tea since the polyphenols are oxidized during the fermentation process, resulting in the changing of the color of the leaves from green to red. The resulting tea is a rich orange/red color and it is this distinctive color that led to the African name *rooibos*, which means "red bush" [McKay and Blumberg, 2007; Erickson, 2003]. The unfermented rooibos, also called green rooibos, contains higher levels of polyphenol antioxidants than its fermented counterpart [Joubert, 1996].

Figure 2.2. Mature rooibos plant [reproduced from Agribussiness in sustainable natural African plant products, Crop profile, www.asnapp.org.za].

2.4 Phytochemicals in rooibos

Polyphenols are one of the most abundant and widely dispersed groups of compounds in the plant kingdom. They are secondary plant metabolites and are often found bound to sugar moieties (glycosides), thereby increasing their solubility in water and allowing forstorage in inactive forms. Polyphenols are formed by two main synthetic pathways: the shikimate pathway and the acetate pathway [Bravo, 1998]. Structurally, polyphenols are characterized by one or more six-carbon aromatic rings and two or more phenolic hydroxyl groups, hence the name polyphenol [Stevenson and Hurst, 2007; Ross and Kasum, 2002]. Plant polyphenols can be grouped in three major classes – lignins, tannins and flavonoids. Flavonoids are the largest class of polyphenols and can be further divided into eight groups based on their skeleton structure: flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidines, chalcones and flavonolignans.

The biosynthesis of flavonoids is initiated by condensation of 4-coumaroyl CoA with three molecules of malonyl CoA to produce a heterocyclic hydrocarbon, chromane, the precursor of all flavonoids [Hodek *et al*. 2002]. A molecule of resorcinol or phloroglucinol, synthesized from the acetate pathway, usually gives rise to the A ring, with a characteristic hydroxylation pattern at C5 and C7. The B ring originates from the shikimate pathway and is usually 4′-, 3′4′-, or ′3′4′5′-hydroxylated [Ross and Kasum, 2002]. The structure of flavonoids is derived from chromane — substitution of its C ring at C2- or C3- with a phenyl group (ring B) to form flavans, and the addition of an oxo-group C4 to form flavanones and isoflavanones (figure 2.3). A double bond between C2 and C3 in the C-ring is often present providing these compounds with quinone-like properties. The C-ring substitution determines whether these flavonoids are assigned as flavones (2-phenyl-group) or isoflavones (3-phenyl group). The well-known group of anthocyanidins is characterized by OH group at C3 an additional double bond in the C-ring to which the colour pigments of leaves, fruits and flowers can be attributed. Chalcones, bi-cyclic aromatic ketones possessing an opened C-ring, act as intermediates in the biosynthesis of flavones and are also classified as flavonoids.

To date, more than 8000 compounds of flavonoid structure have been identified [Pietta, 2000]. Various combinations of multiple hydroxyl and methoxyl substitutions of the basic flavonoid skeleton structure result in the large number of identified compounds.

Flavonoids have various roles in the ecology of plants and plant pigments and odors are attributed to the flavonoid compounds. They act as antioxidants, antimicrobials, photoreceptors, visual attractors and feeding repellants [Hodek *et al*. 2002, Pietta, 2000]. Major sources of flavonoids include fruits, vegetables, tea leaves, soy beans and herbs with almost all plant families having flavonoids in their leaves, stems, roots, flowers and seeds. The main dietary sources of flavonols and flavones are tea and onions, with quercetin being the most abundant flavonol in onions while tea contains significant amounts of both quercetin and kaempferol [Ross and Kasum, 2002, Mesiano *et al*., 1999].

Flavonoids usually occur as glycosides, (e.g glucosides, rhamnoglucosides, rutinosides), although their structures can be more complex, e.g flavonolignans (silybin), catechin esters (epigallocathechin gallate) or prenylated chalcones (xanthohumol) (figure 2.4). In view of the fact that the chemical structures and some activities of several flavonoids are similar to those of naturally occurring estrogens they are frequently classified as phytoestrogens.

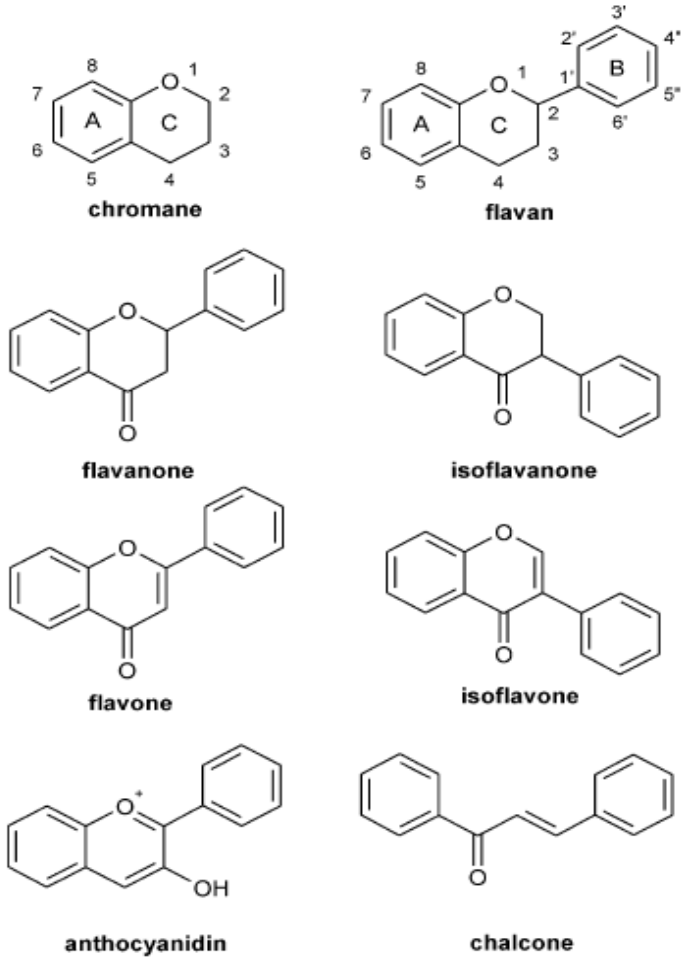


Figure 2.3. Structures of basic flavonoid skeletons [reproduced from Hodek *et al*. 2002].

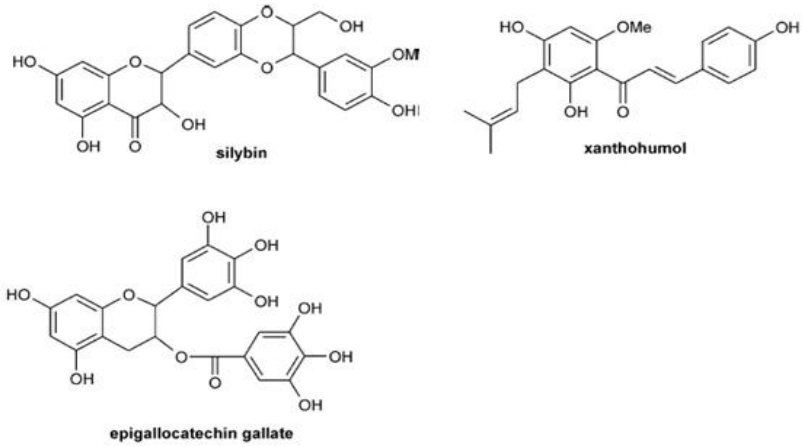


Figure 2.4. Examples of complex flavanoids [reproduced from Hodek *et al*. 2002].

In foods, flavonoids are bound to saccharides as beta-glycosides and it was believed that the absorbtion of flavonoids from the diet were negligible. However, microorganisms in the colon hydrolyze these compounds to aglycones (free flavanoids) which are thought to pass freely into the bloodstream from the gut wall. Recent studies showed that the absorption of quercetin exceeded that of the pure aglycone [Hollman and Katan, 1997]. The two major sites of flavonoid metabolism are the liver and the colon microflora which, in addition to release of aglycones, degrades flavonoids to phenolic acids. Whether flavonoids are more effective in the body as free aglycones or as complex molecules probably depends on the particular flavonoid and its biological activity [Rice-Evans, 2001]. Interest in the possible health benefits of polyphenols, especially flavonoids, has recently increased due to their antioxidant and free-radical scavenging abilities [Ross and Kasum, 2002].

2.4.1 Polyphenols in rooibos

Rooibos contains a wide variety of polyphenols which includes the flavonoids aspalathin, orientin, iso-orientin, rutin, isoquercitrin, vitexin, isovitexin, chrysoeriol, quercetin, luteolin, nothofagin, and (+)-catechin (figure 2.5).

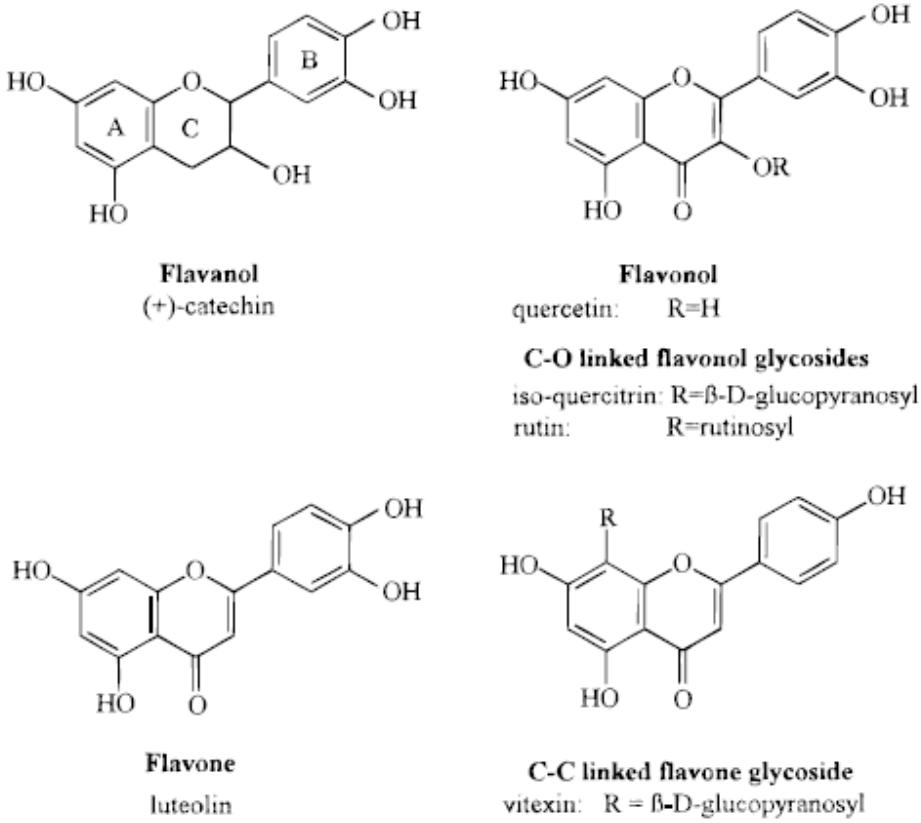


Figure 2.5. Structures of flavonoids present in rooibos tea [Von Gadow *et al*., 1997].

The dihydrochalcones, aspalathin and nothofagin, are the major flavonoids of rooibos tea, constituting approximately 9.3 and 1.03% of the dry plant material respectively. About 43% of the total antioxidant capacity of aqueous extracts of unfermented rooibos [Schulz *et al*., 2003; Von Gadow, 1997] can be attributed to aspalathin with rooibos being the only known natural source of aspalathin reported to date [Joubert, 1996]. Nothofagin is structurally similar to aspalathin except for the hydroxylation pattern of the B-ring (figure 2.6). It was first isolated from the heartwood of *Nothofagus fusca* which is, together with rooibos, currently the only other natural source of nothofagin [Joubert, 1996].

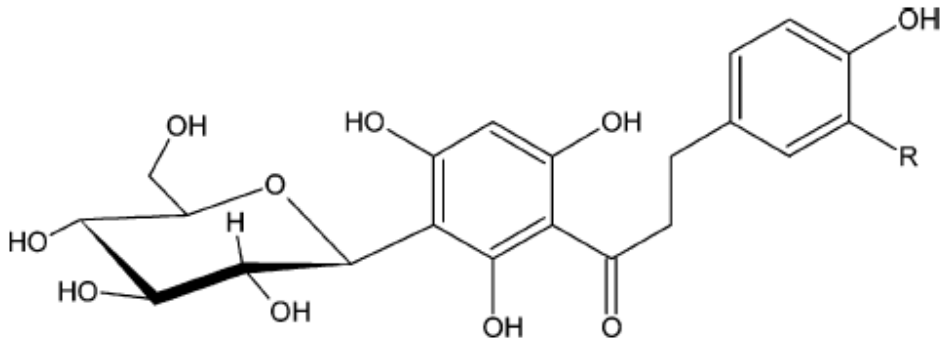


Figure 2.6. Structure of a C-C linked dihydrochalcone glycoside. Aspalathin, R = OH and nothofagin, R = H, found in rooibos [Joubert, 1996].

The 3,4-dihydroxyl arrangement of the B ring, the 2',6'-dihydroxyacetophenone group and the keto-enol transformation of the carbonyl group that stabilizes the radical after hydrogen abstraction are all factors that determine the potency of aspalathin as an antioxidant [Nakamura et at., 2003; Rezk *et al*., 2002]. In studies conducted with lipid radicals, it was shown that the molecular structure and more specifically, the position and degree of hydroxylation of the ring structure of phenolic compounds, determine the antioxidant capability due to the delocalization of unpaired electrons stabilising the formed phenoxyl radical [Gordon and Hudson, 1990; Von Gadow, 1997].

During fermentation, the antioxidant activity of rooibos decreases significantly with the oxidation of aspalathin to dihydro-*iso*-orientin. In a study conducted by Bramati *et al*. 2003, the levels of aspalathin in unfermented rooibos were found to be almost 50 times higher than in fermented rooibos. Less than 7% of the aspalathin content was retained after the fermentation process with other major compounds such as the *C*-glycosyl flavones isoorientin, orientin, vitexin and isovitexin being degraded to a lesser extent. Rutin, the main flavonol-glycoside, is converted to the aglycone quercetin, albeit to a lesser degree as evidenced by its increased level in fermented rooibos (table 2.1). In addition, it was shown that the antioxidant activity of an aqueous extract of unfermented rooibos was 2-fold higher than its fermented counterpart [Bramati *et al*., 2003; Joubert, 1996]. It was concluded that the decrease in antioxidant activity is observed after fermentation can be attributed partly to the oxidation of aspalathin [Von Gadow, 1997]. The higher polyphenol content and antioxidant activity of unfermented rooibos has led to

an increased demand thereof from the global nutraceutical and cosmetic industries both as a herbal tea and a source material used in the preparation of antioxidant-enriched extracts, particularly with regards to aspalathin [Schulz *et al*., 2003].

Rooibos also contains phenolic acids that have been shown to possess antioxidant activity. The majority of these compounds is abundant in nature and is found in fruits, vegetables and whole grains. The phenolic acids in rooibos tea consist of protocatechuic acid, caffeic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, ferulic acid and syringic acid. These compounds have also been shown to possess antioxidant activity [Von Gadow *et al*., 1997].

Table 2.1: Flavonoids detected in unfermented and fermented rooibos aqueous extracts (mg/g ±SD) [reproduced from Bramati *et al*., 2003].

Compound	Unfermented Rooibos	Fermented Rooibos
Isoorientin	3.570 ± 0.18	0.833 ± 0.007
Orientin	2.336 ± 0.049	1.003 ± 0.010
Aspalathin	49.92 ± 0.80	1.234 ± 0.010
Vitexin	0.504 ± 0.002	0.330 ± 0.002
Rutin	1.690 ± 0.14	1.269 ± 0.006
Isovitexin	0.659 ± 0.005	0.265 ± 0.002
Isoquercitrin and hyperoside	0.326 ± 0.006	0.429 ± 0.002
Luteolin	0.020 ± 0.002	0.029 ± 0.001
Quercetin	0.042 ± 0.006	0.107 ± 0.002
Chrysoeriol	0.0079 ± 0.0004	0.022 ± 0.001
Total	59.080 ± 0.59	5.521 ± 0.055

2.4.2 Phytoestrogens

Epidemiological studies comparing disease incidence in countries with excellent health care have shown remarkable geographic differences in the occurrence of types of cancer which are hormone-related type cancers i.e. breast and, prostate cancer, cancers of the digestive tract, as well as in hormone-dependant cardiovascular disease and in the development of post-menopausal-related diseases [Thomas, 1999; Adlercreutz, 2002; Morrisswy and Watson, 2003]. In Asia, the occurrence of these diseases is considerably lower than in Northern Europe and America. These studies thus propose that environmental factors, and in particular dietary

components, play a key role in the development and progression of several cancer types and other hormone related diseases. These protective effects have been attributed to phytoestrogens. Phytoestrogens are particularly abundant in soy products, which comprise a major part of the Asian diet [Ososki and Kennelly, 2003].

Phytoestrogens are plant derived (predominantly legumes and grasses) non-steroid substances, structurally and functionally similar to androgens and estrogens. Broadly defined, they can be divided into three main classes: flavonoids, coumestans and lignans. The most common phytoestrogens are diphenolic chemicals that are structurally similar to natural and synthetic human steroid hormones (figure 2.7) [Mesiano *et al*., 1999; Krazeisen *et al*., 2001; Krurzer and Xu, 1997].

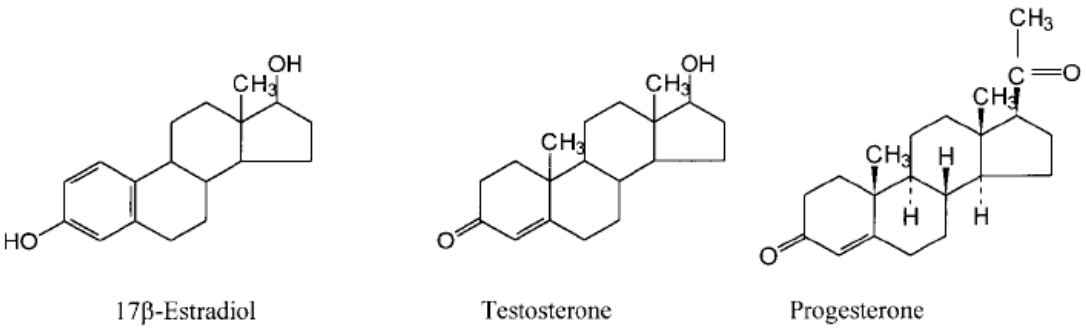
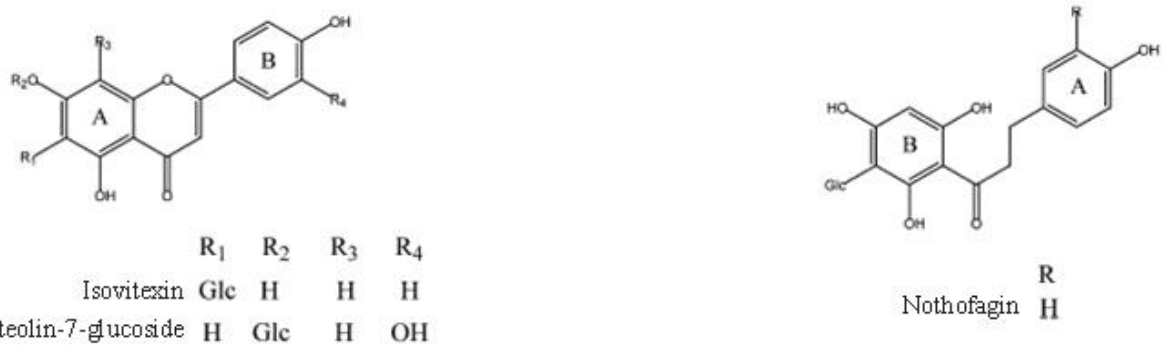


Figure 2.7. Structures of the steroid hormones 17β-estradiol, progesterone and testosterone [Rosenburg *et al*., 2000].

2.4.2.1 Phytoestrogens in rooibos

Since plant-derived phytoestrogens have been shown to exhibit estrogenic activity, Shimamura *et al*. (2006) investigated the phytoestrogenic activity of rooibos. Twenty four known compounds as well as, aspalalinin, were isolated from the leaves of *Aspalathus linearis*. The compounds were evaluated for estrogenic activity using an estrogen ELISA assay and compared to the phytoestrogenic activities of genistein and resveratrol.

The dihydrochalcone, nothofagin, exhibited high activity that was almost equal to that of genistein. However, the estrogenic activity of the other major constituent of rooibos, aspalathin, was found to be less than half of that of nothofagin, suggesting that the hydroxyl group at C-3' reduces its estrogenic activity. Two other compounds, isovitexin and luteolin-7-glucoside, also showed moderate activity (figure 2.8). These results suggested that rooibos may have health benefits due to its mild estrogenic activity [Shimamura *et al*., 2006].



	R_1	R_2	R_3	R_4
Isovitexin	Glc	H	H	H
Luteolin-7-glucoside	H	Glc	H	OH

Figure 2.8. Compounds in rooibos that exhibit phytoestrogenic activity [adapted from Shimamura *et al*., 2006].

2.5 Physiological activity of rooibos

The reported therapeutical properties of rooibos, which include the alleviation of allergies, counteraction of nervous tension, anxiety and depression amongst others, have prompted researchers to investigate various biological activities. The antioxidant activities, anticancer activities, influence on the immune system, and phytoestrogenic activity are some of the areas currently being exploited and these may possibly establish a scientific base for some of the therapeutic properties of rooibos.

2.5.1 Antioxidant activity

Reactive oxygen species (ROS) are formed *in vivo* during normal aerobic metabolism. These unstable molecules are highly reactive due to the loss of an electron and can cause damage to

DNA, proteins and lipids despite natural innate antioxidant defense systems. The accumulation of unrepaired DNA or oxidised biomolecules can lead to cancer, artherosclerosis, diabetes and chronic inflammation. It has been reported that the oxidation of cholesterol by free radicals can lead to clogged arteries, resulting in heart attack and stroke [Ross and Kasum, 2002]. Antioxidants bind free radicals thus preventing oxidative damage. Antioxidants are divided into groups e.g vitamins, carotenoids, minerals and polyphenols. The ability of polyphenols to scavenge ROS has attracted a great deal of attention [Scalbert *et al*., 2005; Erickson, 2003; Kazuno *et al*., 2005].

In the food industry, the lipid peroxidation reaction that occurs during the processing and storage of food, is a major cause of deterioration. This reaction affects the colour, flavour, texture and nutritional value of processed foods [Cheung and Cheung, 2005]. During lipid peroxidation, free radicals "steal" electrons from lipids in cell membranes, thereby causing cell damage. Antioxidants are added to food to delay this process and synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have been used as antioxidants for years. The safety of these synthetic antioxidants has, however, been a cause for concern among consumers. The use of natural antioxidants which inhibit lipid peroxidation, or at least provide protection from the damage caused by free radicals, has received a great deal of attention. In a screening of South African plants for antioxidants, an aqueous extract of rooibos inhibited lipid peroxidation by 90 % [Lindsey *et al*. 2002; Jayaprakasha *et al*. 2001; Yen *et al*. 1997].

Schulz *et al*. (2003) showed a correlation between the aspalathin content and the total antioxidant activity (TAA) of unfermented rooibos when assessed with the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) radical cation-scavenging assay. The TAA of unfermented rooibos was 2.8-fold higher than that of the fermented product. Due to the low levels remaining after fermentation, the contribution of aspalathin to the antioxidant activity of fermented rooibos was very small when compared to the other phenolics present (table 2.2).

Table 2.2: Total polyphenol content, aspalathin content, as well as total antioxidative activity (TAA) of unfermented and fermented rooibos [Adapted from Schulz *et al*., 2003].

Rooibos	Total polyphenols[a]	Aspalathin[b]	Total antioxidant activity[c]
Unfermented	8.12±0.85	4.89±0.93	775.6±130.6
Fermented	4.54±3.58	0.11±0.05	274.5±49.7

a Results expressed as g gallic acid equivalents (GAE) per 100 g dry weight
b Results expressed as g aspalathin per 100 g dry weight
c Results expressed as μmol Trolox equivalents per g dry weight

Other assay methods have also been used to examine the effect of fermentation, processing and preparation conditions on the antioxidant activity of rooibos. The ability of rooibos to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide ($O_2^{\cdot -}$) radicals was tested at five major processing stages by Standley *et al*. (2001). The study found that the decrease in antioxidant and antimutagenic effects was associated with the reduction in the tea polyphenolic content during fermentation [Standley *et al*., 2001].

Von Gadow *et al*. (1997) used the DPPH radical, β-carotene bleaching and automated Rancimat methods to compare the antioxidant activity of aspalathin and other polyphenols present in rooibos tea with the antioxidant reference standards α-tocopherol, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). The phenolic compounds that were tested included the flavonoids vitexin, rutin, quercetin, luteolin, isoquercitrin and (+)-catechin. The antioxidant activity of aspalathin and the other polyphenols were found to be comparable with α-tocopherol and the popular synthetic antioxidants BHT and BHA. The antioxidant activity of the phenolic acids in rooibos tea that was also measured include, in decreasing order of antioxidant activity: caffeic acid, protocatechuic acid, syringic acid, ferulic acid, vanillic acid, p-hydroxybenzoic acid, and p-coumaric acid. Caffeic acid showed similar antioxidant activity compared to quercetin, isoquercitrin and aspalathin, the most potent flavonoids tested [Von Gadow *et al*., 1997].

The ability of flavonoids to scavenge DPPH and superoxide anion ($O_2^{\cdot -}$) radicals were investigated in unfermented and fermented rooibos tea by Joubert *et al*. (2004). In both assays, the unfermented extracts were more effective radical scavengers than their fermented

counterparts. Quercetin was the most potent radical scavenger, with aspalathin, orientin, luteolin and isoquercetin being slightly less active towards DPPH, although aspalathin and quercetin showed the same activity towards superoxide anion radicals. The results of this study corroborated findings of previous investigations [Standley *et al*., 2001; Von Gadow *et al*., 1997] that showed that fermentation decreases the antioxidant capacity of rooibos (Joubert *et al*., 2004].

Studies in cellular systems have also reported the antioxidant activity of rooibos. In human polymorphonuclear leukocytes, quercetin and an aqueous extract of rooibos inhibited the generation of superoxide anion radicals by phorbol myristate acetate [Yoshikawa *et al*., 1990]. Mouse leukemic cells preincubated with rooibos extract exhibited a time- and dose-dependent increase in survival rate after exposure to H_2O_2. However, simultaneous treatment with rooibos did not provide any protection against the cytotoxic effects of H_2O_2 (Ito *et al*., 1992]. The antioxidant activity of freshly brewed and freeze-dried rooibos tea extract on rabbit erythrocyte membrane, rat liver microsome and rat liver homogenate systems was compared by using a linoleic acid autoxidation system. The freeze-dried extract had a strong, dose-dependent effect in the erythrocyte and microsome systems, while the freshly brewed tea displayed strong activity in the rat liver homogenate. Rooibos flavonoids were also tested in the rat liver microsome system and luteolin and quercetin exhibited the highest activity [Hitomi *et al*., 1999].

Marnewick *et al*. (2005) used the formation of thiobarbituric acid reactive substances (TBARS), measured as malondialdehyde (MDA), to determine the protective effects of the ethanol/acetone soluble extracts of unfermented and fermented rooibos against lipid peroxidation, in the presence of Fe_2^+ and absence of hydrogen peroxide by utilizing a rat liver microsomal system. The unfermented extract inhibited the formation of TBARS by 91% and the fermented extract had a similar but less protective (65%) effect [Marnewick *et al*., 2005].

Several animal studies have also been conducted to test the antioxidant capacity of rooibos. The administration of 10µM/kg luteolin, isolated from rooibos, to mice 2 hours prior to γ-ray irradiation significantly reduced lipid peroxidation in mouse bone marrow and spleen [Shimoi *et al*., 1996]. The effect of longterm administration of rooibos tea on lipid peroxidation in the rat

brain was investigated by Inanami and co-workers. Their study found that lipid peroxides, measured with the TBARS assay, were considerably higher in the frontal and occipital cortex, hippocampus and cerebellum of 2 year old rats compared with 5 week old rats. However, there were no significant changes in TBARS of rats given rooibos tea *ad libitum* from age 3-24 months. In addition, signal intensities in the brain of rooibos-treated rats were similar to those perceived in 5 week old rats, while those of untreated 2 year old rats were significantly decreased. These results suggested that the administration of rooibos tea protected several regions of the rat brain against lipid peroxidation accompanying aging [Inanami *et al*., 1995].

The effects of rooibos on the *in vivo* oxidative status and hepatic drug metabolising enzymes in male Fischer 344 rats were tested by giving them water (control), unfermented or fermented rooibos and other teas as their only source of fluid for 10 weeks. Neither one of the rooibos extracts had an effect on the oxygen radical absorbance capacity values in the liver but it did, however, increase the ratio of reduced to oxidized glutathione (GSH/GSSH) [Marnewick *et al*., 2003]. Reduced glutathion is a powerful intracellular antioxidant that plays an important role in stabilizing many enzymes and can be considered as a good marker for the antioxidative capacity in tissue [Prior and Cao, 1999]. Both types of rooibos enhanced the activity of the phase II enzyme, cytosolic glutathione S-transferase alpha (GST-α) by 100% but only unfermented rooibos enhanced the activity of the phase II enzyme, microsomal UDP-glucuronosyl transferase (UDP-GT) by 50%. The induction of phase II hepatic drug metabolising enzymes by rooibos may be a promising tool for chemoprevention against cancer in humans as it is consumed on a regular basis in South Africa and is also gaining popularity as a neutraceutical product on an international basis [Marnewick *et al*., 2003].

The hepatoprotective effects of rooibos were investigated by Ulicn *et al*. (2003) in rats exposed to carbon tetrachloride (CCl_4), a potent pro-oxidant. Histological analyses revealed a regression of CCl_4-induced hepatic steatosis and cirrhosis and a reduction in the production of hepatic malondialdehyde, triacylglycerol and cholesterol as well as plasma activities of aminotransferases, alkaline phosphatase and bilirubin in rats treated with rooibos tea. The observed protection from liver damage observed in the rats may benefit patients with

hepatopathies using rooibos tea as a plant hepatoprotector since the model of CCl_4-induced hepatic fibrosis in the rat imitate many of the features of human liver fibrosis [Ulicn *et al*., 2003].

These studies all suggest that flavonoids found in rooibos, which show antioxidative potency *in vitro*, also act as antioxidants *in vivo* and their radio- and hepatoprotective effects may be attributed to their scavenging potency towards ROS. In the light of these findings it is apparent that flavonoids in rooibos are important as antioxidants in the human diet.

2.5.2 Immune responses

Research has also focused on the immune-boosting properties of rooibos. The anti-oxidants discussed above which are present in rooibos tea could potentially also counteract the effect of free radicals in the decline of the immune system. The immune system plays an important role in maintaining homeostasis by eliminating endogenously formed mutated cells such as virus-infected or tumor cells as well as exogenous invading microbial organisms. The impairment of the immune responses can cause autoimmune diseases and allergy.

Scientists in Japan conducted studies investigating the effects of rooibos tea on antigen-specific antibody production and cytokine generation *in vitro* and *in vivo*. The addition of the tea extract at concentrations of 1 – 100 μg/ml markedly stimulated the primary *in vitro* anti-ovalbumin (anti-OVA) or sheep red blood cell (SRBC) antibody production in response to OVA and SRBC in murine splenocytes. Rooibos tea also increased the generation of interleukin 2 (IL-2) but suppressed the production of interleukin-4 (IL-4) in primed splenocytes. Following oral administrations of rooibos tea extract, the production of antigen-specific antibodies in serum of cyclosporine A (CyA)-treated rats can be restored and the generation of IL-2 stimulated [Kunishiro *et al*., 2001]. These findings suggest that rooibos tea may help in the prophylaxis of diseases related to a severe defect in T helper-1 immune response such as cancer, allergy, AIDS and other infections [Kunishiro and Tai, 2001].

Ichiyama *et al*. (2007) isolated a fraction of rooibos that stimulated the antigen-specific antibody production and interleukin 10 (IL-10) generation *in vitro* [Ichiyama *et al*., 2007]. IL-10 is a cytokine that is produced by T cells —Th1 and Th2, B cells, mast cells and macrophages. Il-10 induces the proliferation and differentiation of B cells, regulates the synthesis of immunoglobulins by B cells and also suppresses the delayed allergy of graft-versus-host disease [Rousset *et al*., 1992; Nagumao and Agematsu, 1998; Wang *et al*., 2002]. Moreover, the continuous ingestion of the fraction also increased the anti-OVA IgM level in sera of OVA-immunized rats [Ichiyama *et al*., 2007].

The therapeutic potential of rooibos for people living with HIV/AIDS is also being exploited. Nakano *et al*. (1997) extracted an acid polysaccharide from rooibos leaves that showed strong anti-HIV activity by inhibiting the binding of HIV-1 to MT-4 cells. Japanese green tea polysaccharides and a hot water extract of rooibos did not show any inhibition. The polysaccharide may thus be involved in the mechanism for virus binding to T cells [Nakano *et al*., 1997].

2.5.3 Chemopreventive potential

Rooibos may also have a role to play in counteracting the development and growth of cancer. Since free radicals have been linked to carcinogenesis, the chemoprotective properties of rooibos are currently being investigated. The effects of different concentrations of rooibos tea extract in medium on the growth and changes of growth parameters of primary cultured chick embryonic skeletal muscle cells were investigated by Lamosova *et al*. (1997)The presence of rooibos tea extract in the culture medium of chick embryonic skeletal muscle cells inhibited cell proliferation and growth in a dose dependent manner. A decrease in the DNA and RNA content of primary cells, fibroblasts and myoblasts in the presence of rooibos tea extract correlated with decreased DNA and protein synthesis measured by [^{3}H] thymidine and [^{3}H] leucine incorporated into DNA and *de novo* protein synthesis. Only a 100% of the tea extract inhibited ornithine decarboxylase (ODC), an enzyme involved in the signal transduction pathway for mitosis. It is hypothesized that the ability of rooibos tea extract to inhibit the growth of chick skeletal muscle

cells can be attributed to its radical scavenging ability, which prevented ODC from triggering mitosis in the presence of free radicals [Lamosova *et al*., 1997].

Komatsu *et al*. (1994) showed that rooibos tea extract suppress the X-ray induced oncogenic transformation of mouse embryo fibroblast cells in a dose- and time-dependant manner [Komatsu *et al*., 1994]. The number of chromosome aberrations in Chinese hamster ovary (CHO) cells induced by benzo[a]pyrene (B(a)P) or mitomycin C (MMC) is significantly suppressed by rooibos tea. The consumption of rooibos tea may thus possibly suppress the mutagenic activity in humans of certain potent mutagens [Sasaki *et al*., 1993].

The radioprotective effects of rooibos tea were investigated and it was shown by Shimoi *et al*. (1996) that the frequency of micronucleated reticulocytes (MNRET), which are cells with damaged DNA that may lead to cancer, is reduced by a single gastric intubation of the tea at 1 ml per mouse 2 hours prior to γ-ray irradiation. A flavonoid containing fraction isolated from rooibos that included luteolin and quercetin, was found to be the most anticlastogenic [Shimoi *et al*., 1996].

Various plant-derived beverages, including green, black and rooibos tea, were tested by Edenharder *et al*. (2002) for their protective effects against genotoxicity induced by 2-acetylaminofluorene (AAF) or 2-amino-1-methyl-6- phenylimidazo [4,5-b]pyridine (PhIP) in V79 cells of the Chinese hamster expressing rat cytochrome P450 dependent monooxygenase 1A2 (CYP1A2) and sulfotransferase 1C1 (SULT1C1). The genotoxicity of AAF was strongly reduced by green, black and rooibos tea (IC_{50} 0.20 %, 0.19 %, 0.68 % v/v, respectively). The genotoxicity of PhIP was strongly reduced in a dose-dependant manner by green tea (IC_{50} = 0.20%) while black (IC_{50} = 1.25%) and rooibos tea (IC_{50} = 1.29%) were less active [Edenharder *et al*., 2002].

The *Salmonella typhimurium* mutagenicity assay was used by Marnewick *et al*. (2000) to examine the antimutagenic properties of fermented and unfermented rooibos tea. Aqueous extracts of both fermented and unfermented rooibos tea exhibited significant inhibition towards

2-acetylaminofluorene (AAF) and aflatoxin B1 (AFB_1)-induced mutagenesis in tester strains TA98 and TA100 in the presence of metabolic activation. However, a weaker inhibitory effect was observed against the direct acting mutagens, methyl methanesulfonate (MMS), cumolhydroperoxide (CHP), and hydrogen peroxide (H_2O_2) using TA102, a strain designed to detect oxidative mutagens and carcinogens. Unfermented rooibos exhibited the highest protective effect against AAF-induced mutagenesis [Marnewick *et al*., 2000]. Marnewick *et al*. (2004) subsequently investigated *ex vivo* antimutagenic activity in liver cytosolic fractions of tea-treated rats. Fermented and unfermented rooibos protected against aflatoxin B_1 (AFB_1)-induced mutagenicity in *Salmonella* strain TA100 while unfermented rooibos also protected against AAF-induced mutagenicity in *Salmonella* TA98. Hepatic microsomal fractions of rooibos-treated rats significantly inhibited the mutagenic response of AFB_1 [Marnewick *et al*., 2004]. Rooibos extracts may interfere with cytochrome P450-mediated metabolism of carcinogens that require metabolic activation or the rooibos compounds can directly interact with the promutagens and/or the active mutagenic metabolites, based on the results of these studies [Marnewick *et al*., 2000].

Quercetin and luteolin, two of the flavanoids in rooibos tea, have strong antioxidant activity and are found in many fruits and vegetables. *In vitro* studies have demonstrated that these compounds can cause apoptosis of cancer cells and inhibit the proliferation of thyroid cancer cells. In a model of pancreatic cancer, quercetin decreased primary tumor growth and prevented metastasis [Lee *et al*., 2002; Mouria *et al*., 2002; Yamashita and Kawanishi, 2000; RoyChowdhury *et al*., 2002; Yin *et al*., 1999; Mori *et al*., 2001; Mutoh *et al*., 2000]. The inhibition of cyclooxygenase-2 (COX-2) expression in colon cancer cells by quercetin may possibly prevent colon cancer. However, although these studies show that quercetin and luteolin have anticancer properties scientists have yet to determine whether the concentration of either of these compounds in rooibos tea, as well as their absorption, is such that the compounds could have beneficial physiological effects.

Topical applications of methanol fractions of fermented and unfermented rooibos were investigated in a two-stage mouse skin carcinogenesis assay. The application of the tumour

initiator, 7,12-dimethylbenz[a]anthracene (DMBA) and, one week later, the tumour promoter, 12-O-tetra-decanoylphorbol-13-acetate (TPA), was followed by the application of the tea extracts. Skin tumorigenesis was significantly reduced by both fermented (75%) and unfermented (60%) rooibos. These studies showed that a variety of phenolic compounds exhibit chemoprotective properties by disrupting the different stages of carcinogenesis [Marnewick *et al*., 2005].

2.5.4 Role of phytoestrogens in human health

Phytoestrogens, naturally occurring non-steroidal plant compounds exhibiting structural similarity with 17B estradiol have relatively weak estrogen-like activity, exerting either estrogenic and/or antiestrogenic effects. However exposure to high levels for long periods of time may result in significant endocrine disruption [Seifert *et al*., 2004]. There are various reports documenting the presence of endocrine-disrupting chemicals (EDCs) in our environment. These include food contaminants, pharmaceutical and industrial products which can have a disruptive effect on the development, programming and/or normal homeostatic functions of the endocrine system, especially in the reproductive tract [Fisher, 2004]. Phytoestrogens may interfere with the complex human endocrine system in three possible ways. Phytoestrogens might mimic endogenous hormones: 1) at the hormone receptor, exerting agonistic or antagonistic effects; 2) at key enzymes of hormone metabolism, affecting the level of active steroids; or 3) may have diverse non-hormonal effects (Kurzer and Xu, 1997].

Dietary phytoestrogens have widespread clinical effects and are reported to reduce cancer risk, play a role as antioxidants and free radical scavengers, reduce serum cholesterol, induce cellular differentiation and inhibit angiogenesis [Knight and Eden, 1996; Messina *et al*., 1994; Jha *et al*., 1985., Anderson *et al*., 1995; Constantinou and Huberman, 1995; Fotsis *et al*., 1993]. Genistein and daidzein, two phytoestrogens abundant in soy-based foods, have been found by Mesiano *et al*. (1999) to increase androgen and decrease glucocorticoid production by cultured human adrenal cortical cells. These effects occurred at concentrations that were within the reported range for infants and adults consuming a soy-rich diet. However, blood concentrations and tissue

levels may differ markedly. Controlled *in vivo* studies would firmly establish whether phytoestrogens do indeed modulate adrenal cortical steroid production. Their data indicates that genistein and daidzein decreased cortisol synthesis by suppressing CYP21 enzymatic activity. The expression of the gene encoding this enzyme was not affected which suggests that phytoestrogens modulate adrenal steroidogenic activity via nongenomic mechanisms, the most likely being by directly modulating the steroidgenic enzyme activity.

In addition to decreasing cortisol synthesis, genistein and daidzein altered androgen production by increasing dehydroepiandrostenedione (DHEA) and DHEA-S synthesis. DHEA is a precursor of androgenic and estrogenic endogenous sex steroids. Thus, phytoestrogens may decrease cortisol synthesis by suppressing the activity of P450c21 and, as a result, indirectly increase total estrogen and/or androgen levels by increasing DHEA production by shunting metabolites away from the glucorticoid biosynthesis pathway. It is therefore possible that the consumption of foods containing phytoestrogens may alter adrenocortical function by decreasing cortisol and increasing androgen production. Thus, some of the estrogenic actions of dietary phytoestrogens may be mediated via their stimulation of adrenal androgen biosynthesis [Mesiano *et al*., 1999].

Ohno *et al*. (2002) had similar results and also demonstrated that genistein decreases serum corticosterone levels in human adrenal H295R cells by inhibiting 3β-hydroxysteroid dehydrogenase (3β-HSD) and CYP21-hydroxylase activity. Furthermore, phytoestrogens have been shown to suppress the activity of fungal 17β-hydroxysteroid dehydrogenase [Kristan *et al*., 2005]. Supornsilchai *et al*. (2005) also studied the effect of a phytoestrogen, resveratrol, on rat adrenal steroidogenesis. Resveratrol is found in grapes, mulberries and peanuts, all of which are regularly consumed by humans. They concluded that resveratrol suppresses corticosterone production by rat adrenocortical cells *in vitro*, *in vivo* and *ex vivo* by inhibiting cytochrome P405 c21-hydroxylase. Further studies are necessary to evaluate the significance of the results in the pathophysiology of endocrine disruption [Supornsilchai *et al*., 2005].

Several studies described the potential activities of phytoestrogens as endocrine disruptors in males. It has demonstrated that the ingestion of high levels of phytoestrogens in various animal

species can have adverse effects on reproductive endpoints, including fertility. It has also been shown that exposure to high doses of phytoestrogens during development can affect brain differentiation and reproductive development negatively in rodents.

There is a lack of information regarding the possible effects of high doses of phytoestrogens in infants and should receive attention so that possible risks or benefits can be determined. In adults, there is currently no data suggesting that consumption of phytoestrogens at levels normally found in the diet is likely to be harmful. In fact, as previously mentioned, epidemiological studies suggest foods containing phytoestrogens may have a beneficial role in offering protection against a number of chronic diseases and conditions.

Dietary intervention studies in women indicate that soy and linseed may have beneficial effects regarding breast cancer and may help to relieve postmenopausal symptoms. In the case of osteoporosis, tentative evidence suggests phytoestrogens may have similar effects in maintaining bone density to ipriflavone, a related pharmaceutical compound. It appears that soya also has beneficial effects on blood lipids which may help to reduce the risk of cardiovascular disease and atherosclerosis. However, in general, there is little evidence that links these effects directly to phytoestrogens. Soy and linseed contain many other compounds that are biologically active in various experimental systems which may explain the effects observed in humans. Dietary phytoestrogens may have a preventive role in several types of chronic diseases including certain cancers. However, at present the evidence is not sufficient to recommend particular dietary practices or changes. Supportive findings from studies are an indication of the need for further research to clarify the biological activities of phytoestrogens in humans [Humfrey, 1998].

2.5.5 Interaction of flavonoids and Cytochrome P450 enzymes

The heme-containing mixed-function oxidases cytochrome P450 enzymes (P450s) play a key role in the metabolism of hydrophobic endogenous substrates such as steroids, and ingested xenobiotics, foreign compounds such as drugs, food components and carcinogens. Generally, P450s convert xenobiotics to less toxic products but the reactions frequently involve the

formation of reactive intermediates or allow the leakage of free radicals capable of causing toxicity. The interaction of these proteins with flavonoid compounds occur in at least three ways: 1) the biosynthesis of several CYPs is induced by flavonoids; 2) the enzymatic activities of P450s are altered (inhibited or stimulated) by flavonoids and; 3) several P450s metabolize flavonoids. In order to understand flavonoid metabolism in humans, their induction of P450s, their capacity to bind to P450s and P450-mediated conversion of these compounds need to be considered.

Since P450s are also steroidogenic enzymes, particular flavonoids are capable of targeting steroid biosynthesis due to their interaction with these enzymes. Flavonoid activity is believed to be connected with lowered incidence of estrogen-promoting cancers. Since the flavonoid structures resemble that of estrogens, certain classes of flavonoid compounds are assigned as phytoestrogens. Flavonoids exhibit estrogenic or anti-estrogenic effects in organisms due to the fact that like natural estrogens, these flavonoids are able to bind to estrogen receptors and modulate their activity. In addition, aromatase (CYP19), a crucial enzyme of estrogen biosynthesis, and/or steroid dehydrogenases, is inhibited by phytoestrogens [Kao *et al*., 1998; Lee *et al*., 1996]. The binding of flavonoids to receptors and the inhibition of CYP19 trigger complex changes that induce a shift in the overall hormonal balance of an individual. Flavonoids have been shown to prevent bone loss and decrease osteoporotic effects and other menopausal symptoms [Messina, 1999]. Having an anti-estrogenic effect, flavonoids exhibit anti-cancer activity in tissues exposed to sex hormones such as the breast and prostate gland [Nagata *et al*., 2001]. As steroidogenic enzymes inhibitors and estrogens receptor modulators flavonoids have thus been extensively studied for use in the prevention and treatment of some cancers as well as menopausal symptoms.

Flavones and flavanones generally have higher CYP19 inhibitory activity than isoflavones and isoflavanones. The presence of 4′-hydroxyphenyl group at the C3 position greatly reduces the ability of isoflavones to bind to and inhibit CYP19 [Ibrahim and Abdul-Hajj, 1990]. The conversion of the C2, C3 double bond in flavones to a single bond (flavanones) does not have a significant effect on the binding of the compound to the aromatase. Computer modeling studies

showed that flavonoids bind to the active site of CYP19 in the orientation in which rings A and C mimic rings D and C of the androgen substrate. The study underlines the significance of various hydroxyl groups for flavonoid interaction with CYP19. The presence of the C4 oxo-group, that is approaching the heme iron of CYP19 in the model, seems to be an important factor for inhibition. The reduction of the C4 oxo-group to a hydroxyl group results in a decrease in inhibitory potency. The role of hydroxyl groups in flavonoid structures is, however, paradoxical. It is expected that the more hydroxyl groups present, the higher the polarity of the derivative and a subsequent lower binding capability for CYP19. The presence of hydroxyl groups in certain positions is, however, a prerequisite for high inhibitory potency. The presence of a hydroxyl group to C7 of flavone increases the inhibition activity of the compound 20 times compared to a non-substituted flavone. Conversely, the presence of single hydroxyl groups at C3, C5 or C6 was found to significantly reduce inhibitory activity. Interestingly, the further away the hydroxyl group is located from the C4 oxo-group, the higher the inhibitory efficiency of the compound. These data were obtained using different experimental systems that include human expressed CYP19, placental microsomes, microsomes containing recombinant human CYP19 and cytochrome P450 NADPH reductase [Kao *et al*., 1998].

Several aspects thus need to be taken in consideration when the inhibitory potency of compounds are determined — metabolism by other enzymes present in preparations, transport mechanisms of cells and membrane solubility of the flavonoids. In China and Japan, epidemiological studies and *in vitro* laboratory experiments support the belief that the inclusion of flavonoids in human diet can reduce the risk of various cancers, especially hormone-dependent breast and prostate cancer. Flavonoids present in plant derived food and beverages are assumed to be at least partly responsible for their cancer-prevention effects [Ueng *et al*., 1997; Hodek *et al*., 2002].

2.5.6 Potential health/therapeutic applications of rooibos

Rooibos has been used as a folk remedy for many years and some traditional remedies have indicated rooibos to treat asthma, colic, eczema, headache, nausea and mild depression. Rooibos

has also been used as an antihypertensive agent, immune stimulant, laxative, sedative and spasmolytic agent as well as for the treatment of allergies, atherosclerosis and diabetes. Rooibos distributors often suggest that it may remedy these ailments although these claims have not been scientifically verified. Many of these health claims originate from the use of rooibos by Annekie Theron in 1968 who found that rooibos eased her infant's colic.

Anxiety, depression, nervous tension, atherosclerosis and diabetes are all factors associated with abnormal high cortisol levels, impacting negatively on normal endocrine function [Morton, 1983; Bramati *et al*., 2003; Reiche *et al*., 2004; Charmandari *et al*., 2005; Black, 2006]. For the use of herbal medicines to be recognized and accepted by the community at large, these therapeutic claims need to be verified scientifically.

2.6 Summary

Rooibos has been used for centuries and enjoys a strong positive consumer image in South Africa. The rising consumption of rooibos tea both locally and internationally can be attributed to its fruity, sweet taste and its caffeine-free, low tannin and antioxidant-rich status. Rooibos appears to be safe with no side effects. Unfermented rooibos is characterized by a higher amount of polyphenols than the traditional fermented rooibos and also displays higher antioxidant and antimutagenic capabilities *in vitro*.

Although the anti-oxidative, -inflammatory, and -microbial activities of rooibos have been scientifically proven, there are still claims that have not been substantiated with scientific proof, such as the reported alleviation of anxiety, depression, nervous tension, atherosclerosis and diabetes by rooibos. Confirmation of the effect of rooibos on the different homeostasis systems of the body will provide further evidence for the acceptance or dismissal for the health promoting properties of rooibos. In the following chapters the stress response, with specific focus on adrenal cytochrome P450 enzymes, will be discussed. The effect of rooibos on adrenal steroidogenesis will be investigated to provide possible scientific evidence for the stress relieving properties rooibos is reported to have.

Chapter 3
Physiology of the stress response

3.1 Introduction

Life exists by maintaining a relatively stable internal environment, termed homeostasis. Homeostasis is constantly challenged by stressors — both intrinsic and extrinsic, real or perceived undesirable factors. Stress, the response of the body to any factor that disrupts or threatens to disrupt homeostasis, can be defined as a state of disharmony or threatened homeostasis [Sherwood, 2004; Charmandari *et al*., 2005]. Hans Selye, a pioneer in addressing general principles of physiology and pathophysiology in the exploration of stress, was the first to show that stress or harmful agents initiate a reaction in the body. He defined stress as ‘‘the nonspecific response of the body to any demand’’. The function of the stress response is to maintain stability or homeostasis and long-term activation of the stress system can have adverse and even lethal effects on the body [Selye, 1936; Baoa *et al*., 2008].

Factors that induce a stress response range from a variety of stressors: physical stressors such as pain, intense exercise, hemorrhagic shock, surgery, extreme heat or cold; psychological or emotional stressors such as anxiety, fear, sorrow, abuse; social stressors such as personal conflicts, change in lifestyle and factors related to the environment at home or work [Reichea *et al*., 2004; Sherwood, 2004]. The way in which a person perceives a situation can also determine a person’s ability to adjust to repeated stress. The same stressful stimulus may cause major behavioral and physiological changes in one individual but only a minimal response in another [Leonard, 2005].

In response to a stressor, the HPA axis is activated and hormonal secretion from the adrenal gland is stimulated. A variety of adaptive responses at the neuroendocrine, autonomic and behavioral levels are orchestrated by the ensuing rise in cortisol plasma levels. The state of disturbed homeostasis can be termed eustress, or the acute stress response, providing that the

adaptive responses are adequate to restore homeostasis. The Greek prefix *eu*- indicates a beneficial effect to the individual. Not only is sufficient activation an important characteristic of eustress but a tightly controlled termination of the stress response after a particular stressor has ceased and homeostasis has been reinstated, is just as important (figure 3.1). If, however, the activation of the neuroendocrine systems is not appropriately terminated, chronic hyperactivation of the HPA axis may include maladaptive states, termed distress that can be harmful. The Greek prefix *dis*- indicates the detrimental effects to the individual. Chronic activation of the stress response may lead to various disorders as a result of an increased or prolonged secretion of cortisol [Charmandari *et al*., 2005; Habib *et al*., 2001; Chrousos and Gold, 1992].

Whether a stressor causes eustress or distress depends on its duration and intensity and mostly on the subject's genetic predisposition, life history and ontogenetic stage. Together, these factors determine how the stressor is perceived and interpreted by the individual and clarify why stress is associated with emotions such as anxiety and sadness.

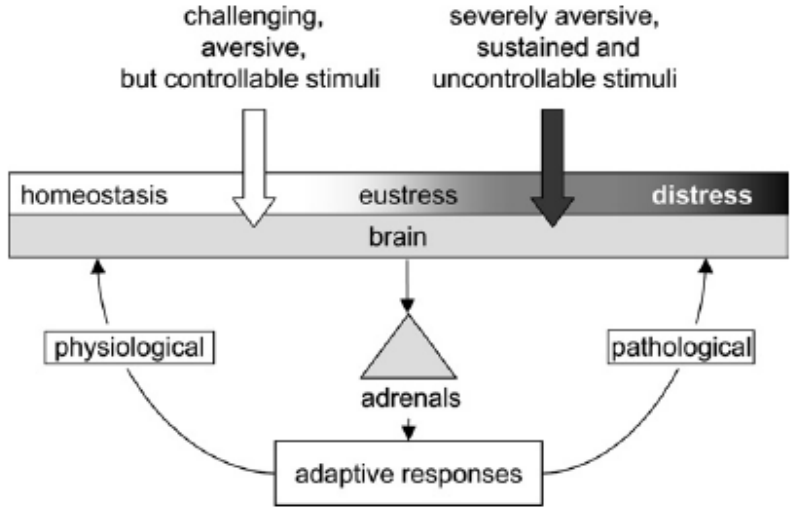


Figure 3.1. Concept of eustress and distress [reproduced from Engelmanna *et al*., 2004].

3.2 HPA axis

The HPA axis is a complex regulatory system that consists of the hypothalamus, pituitary and adrenal cortex, as well as the related regulatory inputs, releasing factors and hormones. The HPA axis integrates neuronal and endocrine functions and has far reaching adaptive effects on metabolism as well as the immune and central nervous systems [Watson and Mackin, 2006; Kerna *et al*., 2008].

In response to a stressor, the neurosecretory cells of the paraventricular nucleus (PVN) of the hypothalamus secrete corticotrophin-releasing hormone (CRH), arginine vasopressin (AVP) and oxytocin (OT) into the microportal circulatory system of the pituitary stalk [Watson and Mackin, 2006; Jacobson, 2005]. CRH and AVP bind to, and activate, specific receptors, CRF_1 and V_{2b} respectively, on anterior pituitary corticotrophes. CRH is the principal hypothalamic stimulus of the pituitary-adrenal-axis regulating the secretion of pro-opiomelanocortin (POMC) gene products which include adrenocorticotrophic hormone (ACTH) and β-endorphin from the anterior pituirary corticotrophs as well as various other hormones such as α, β, and χ melanocyte-stimulating hormone (MSH) [Chrousos and Gold, 1992]. POMC undergoes a series of posttranslational processing steps to produce these tissue specific final products [Kamal *et al*., 2001]. ACTH promotes the release of the glucocorticoid hormones from the adrenal cortex, mainly cortisol in humans (figure 3.2) [Baoa *et al*., 2008].

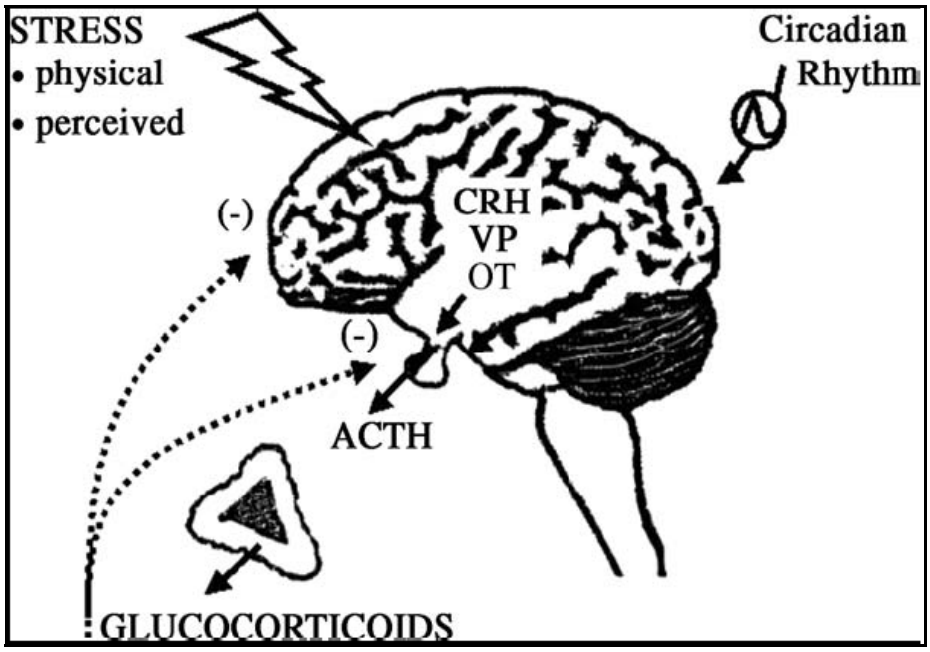


Figure 3.2. Major stimulatory and inhibitory regulators of the HPA axis. Solid lines indicate stimulation and dotted lines indicate inhibition [reproduced from Jacobson, 2005].

Although AVP and OT are potent synergistic factors of CRH in the stimulation of ACTH secretion, they have little influence on the secretion of ACTH on their own [Charmandari *et al*., 2005; Jacobson, 2005]. CRH and AVP are secreted in the portal system in a circadian, pulsatile manner. In nonstressful situations, CRH and AVP are secreted with a frequency of about two to three episodes per hour. An increase in the amplitude of CRH and AVP pulses occur under resting conditions in the early morning hours, which results in the increase of ACTH and cortisol secretion. These diurnal variations are disturbed by changes in lighting, feeding schedules, activity and are disrupted by stress.

During acute physical stress, the amplitude and synchronization of CRH and AVP pulsations increase, and as a result the production of ACTH and cortisol also increases [Tsigos and Chrousos, 1994]. AVP, originating from magnocellular neurons, is also secreted into the hypophyseal portal system. This type of stressor also influences the secretion of other factors such as angiotensin II, various cytokines and lipid mediators of inflammation, all of which

increase the activity of the hypothalamic, pituitary, and/or adrenal components of the HPA axis [Wilson and Mackin, 2006].

Humans and most mammals have two adrenal glands embedded in a capsule of fat, located atop each kidney. Each adrenal consists of two functionally different endocrine organs, the cortex and the medulla. The conventional view that the two different endocrine tissues are clearly separated into an outer steroid-producing cortex and a central medulla, is however, an oversimplification. Medullary cells present in the cortex were first observed 30 years ago in rats [Ehrhart-Bornstein *et al*., 1998; Kmiec, 1968]. This occurrence was not readily accepted but today it is widely acknowledged that medullary cells can be found in all zones of the adrenal cortex, either by radiating through the cortex or dispersed as islets or single cells. On the other hand, cortical cells are also located in the medulla where they may form islets that either maintain some contact with the rest of the cortex or may be surrounded by medullary tissue [Bornstein *et al*., 1994]. This intimate combination of the two cell types forms the basis for possible interactions between the two endocrine systems.

Being embryologically derived from different tissue, the adrenal cortex and medulla secrete hormones belonging to different chemical categories of which the function, mechanism of action and regulation are completely different. The outer layers composing the adrenal cortex secrete a variety of steroid hormones and the inner portion, the adrenal medulla, secretes catecholamines [Ehrhart-Bornstein *et al*., 1998; Sherwood, 2004].

Cortisol is a major stress hormone acting on various organs and areas in the brain. The central and peripheral effects of cortisol include the coordination of circadian events, such as the sleep/wake cycle, the coping with, adaptation to and recovery from stress as well as promoting learning and memory processes. Prolonged exposure to elevated cortisol levels, as in chronic stress, has been shown to have detrimental effects on human health [Vermetten and Bremner, 2002].

The effects of cortisol are mediated by two types of intracellular, specialized steroid receptor family subtypes, the high-affinity, type I mineralocorticoid receptor (MR) and the low affinity, type II glucocorticoid receptor (GR). Cortisol easily diffuses through the cellular membrane where, upon binding to these receptors, it promotes their translocation into the nucleus. Inside the nucleus, the activated receptors either interact with other transcription factors or bind to specific DNA and promote the expression of various genes. The MR is expressed predominantly in the hippocampus, amygdale and pituitary, all of which are relative to cognition. Due to the much higher affinity of the MR for cortisol than that of the GR, it has been proposed that the MR is most likely to be saturated at normal circulating glucocorticoid levels. On the other hand, the GR has a much wider distribution in the brain and periphery and, due to its low affinity for cortisol, it is activated when circulating glucocorticoids levels are high, e.g at the circadian peak or after a specific stressful episode [De Kloet, 2004].

Corticosteroid production in steroidogenic tissues is under the control of tropic hormones secreted from the pituitary gland. After binding to their associated G protein-coupled receptors, the tropic hormones activate adenylyl cyclase, which in turn induces the synthesis of cAMP-dependant protein kinase A (PKA). The cAMP-PKA regulation of steroid production can be divided into two phases: the acute and the chronic phase. The acute phase is completed within a few minutes or lasts up to hours and in this period there is a rapid production of steroids in response to immediate needs. The availability of cholesterol is increased by the activation of cholesterol esterases and carrier proteins, which regulate the biosynthesis of glucocorticoids to combat stressful situations. Included in this phase is the rapid synthesis of aldosterone to regulate blood pressure. The chronic phase is characterized by the stimulation of the expression of genes encoding steroidogenic enzymes, such as the cytochrome P450 enzymes, which occurs after several hours [Grossman, 1998].

3.3 The regulation of the HPA axis in response to stress

The activity of the HPA axis is highly regulated in the hippocampus, amygdale and pituitary and these areas of the brain are actively involved in the feedback regulation of the HPA axis as

secretory cells in the PVN receive neuronal inputs from these regions. In addition, the HPA axis has an auto regulatory mechanism that is crucial for the maintenance of its intrinsic homeostasis. This mechanism is mediated by the binding of cortisol to the GR in the HPA axis and hippocampus [Watson and Mackin, 2006; Baoa *et al*., 2008].

The hypothalamus, anterior pituitary and adrenal glands (HPA axis) represent a controlling loop by which secretion of corticosteroids is controlled in response to environmental stimuli and physiological changes in the body. In response to stress, the hypothalamus is stimulated to secrete CRH, which is transported to the anterior pituitary, stimulating ACTH secretion from pituitary corticotrophs. ACTH reaches the adrenal glands via the systemic circulation to boost the secretion of corticosteroids which include mineralocorticoids and glucocorticoids. Stimulation of the adrenal is regulated by a short negative feedback loop in which increased ACTH downregulates the secretion of CRH (figure 3.3). Glucocorticoids secreted from the adrenal cortex also exert a negative feedback on the pituitary and hypothalamus in a similar fashion to downregulate CRH and ACTH secretion, respectively [Stocco, 2001; Guo *et al*., 2003]. The regulation of steroid hormones biosynthesis, the enzymes involved and their intracellular location, will be discussed in detail in the following chapter.

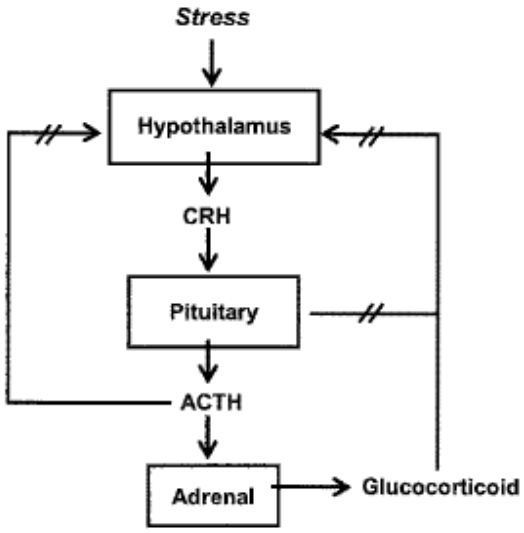


Figure 3.3. Components the HPA axis [reproduced from Guo *et al*., 2003].

The activity of the HPA axis is regulated in a different manner during acute and chronic stress [Leonard, 2005]. During acute stress, the activation of the HPA axis produces a temporary increase in plasma glucocorticoids. This continues during, and shortly after the experience of the stressful stimulus and includes a rapid desensitisation of glucocorticoid receptors in the brain, resulting in an elevation in circulating cortisol, with a concomitant resistance to feedback inhibition. Termination of the acute stress response ensues with the decrease in the release of CRF from central neurons, resulting in a decrease in glucocorticoid concentration. The normal feedback inhibitory system is restored together with the normalization of the density of glucocorticoid receptors.

During chronic stress, the HPA response to elevated glucocorticoid levels is preserved [Jacobson, 2005]. The changes observed in the HPA axis, resulting from chronic stress, vary according to whether the subject has developed coping strategies. An individual who has adapted to chronic stress has higher basal plasma glucocorticoid and ACTH levels. In response to a stressor, these levels do not increase as dramatically when compared to changes observed during acute stress [Kant *et al*., 1987; Leonard, 2005]. In contrast, chronic stress may often lead to hypersecretion of adrenal glucocorticoids and sustained activation of the central and peripheral sympathetic systems [Irwin *et al*., 1991]. These changes occur due to the desensitisation of the glucocorticoid receptors and resistance to feedback inhibition. Chronic stress is thus associated with a hypersecretion of CRF and a decreased sensitivity to feedback inhibition by cortisol [Leonard, 2005]. Stress, depending on whether it is acute or chronic, can influence a number of physiological processes.

3.4 Physiological responses during stress

During stress, behavioral and physical changes occur which improve an individual's ability to cope during a stressful episode. The brain is focused on the perceived threat and behavioral changes, which include increased arousal, alertness and improved cognition, occur. Endocrine functions such as appetite, feeding and reproduction are inhibited to save energy and a concomitant physical adaptation takes place encouraging the redirection of energy. An increase

in cardiac output and respiration occurs and oxygen and nutrients are redirected to the CNS and the stressed body sites.

Restraining forces that prevent an over response from the components of the stress system are activated during stress. If these forces are excessive or unsuccessful in containing the components of the stress response, adaptive changes may become chronically deficient or excessive. Chronic activation of the stress system due to an increased or prolonged secretion of cortisol may lead to the development of physiological and psychological disorders [Kamal *et al*., 2001; Charmandari *et al*., 2005].

The transient activation of the HPA axis during the acute stress response is beneficial for restoring homeostasis. However, if the activation is not terminated, chronically elevated cortical levels might become detrimental to an individual. The effects of chronically elevated glucocorticoid levels (figure 3.4), mainly cortisol, on the immune system, glucose metabolism, growth and blood pressure, as well as the psychiatric disorders associated with stress, are discussed in the following section.

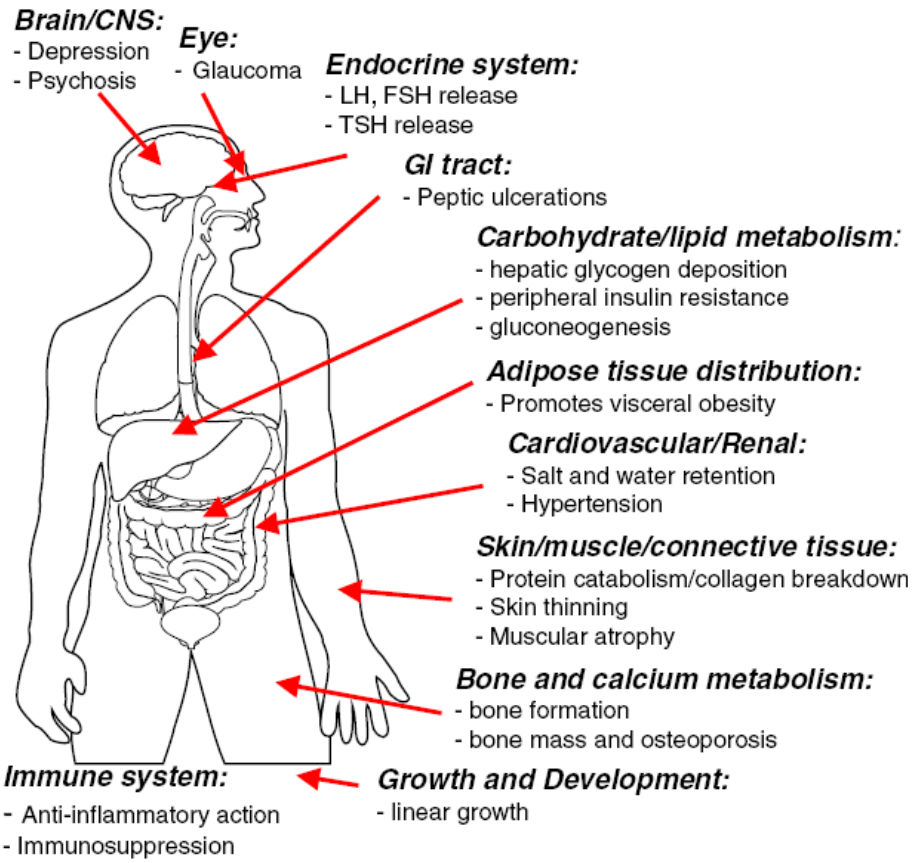


Figure 3.4. Main sites of action of glucocorticoids in individuals with arrows indicating the diverse effects of cortisol upon various tissues [reproduced from Arlt and Stewart, 2005].

3.4.1 Immune response

The incentive to develop a series of highly effective pharmacological glucocorticoids to treat several autoimmune and inflammatory conditions stems from the fact that glucocorticoids suppress immune responses. Chronic stress influences the immune response due to the fact that nearly all the components of the response are inhibited by cortisol. In blood, the lymphocyte count is significantly reduced by redistributing lymphocytes from the intravascular compartment to the spleen, lymph nodes and bone marrow. Glucocorticoids have a direct influence on both T and B lymphocytes by inhibiting immunoglobulin synthesis and stimulating lymphocyte apoptosis [McKay and Cidlowski, 1999].

Inhibition of the action of nuclear factor kappa B (NF-κB) mediates the inhibition of cytockine production by lymphocytes. NF-κB plays a vital and general role in stimulating cytokine gene transcription; glucocorcorticoids can prevent nuclear translocation by binding directly to NF-κB and also inducing NF-κB inhibitors which sequester NF-κB in the cytoplasm, thereby inactivating its effect. Additional anti-inflammatory effects caused by glucocorticoids involve the inhibition of monocyte differentiation into macrophages and macrophage phagocytosis and cytotoxic activity. The inhibition of histamine and plasminogen activators by glucocorticoids reduces the local inflammatory response [Graham and Tucker, 1984].

Cytokines are soluble mediators that are released by various cells both at the periphery by macrophages and lymphocytes. Depending on the functional profile of the secreting T-helper cells, the production of cytokines is divided into two broad categories: type 1 helper (Th1) cells and type 2 helper (Th2) cells. Th1 cells usually mediate the cellular immune response through the activities of cytotoxic lymphocytes, natural-killer (NK) cells and macrophages and include the production of the cytokines interferon γ, TNF-α and IL-2. Th2 cells augment immune reactions mediated by antibodies and include the production of IL-4, IL-5, IL-6 and IL-10. Th1 and Th2 cells can be cross-inhibitory, with IL-4 and IL-10 released by Th2 cells exerting anti-inflammatory effects that suppress the activity of Th1 cells and stimulate the Th2 cell and humoral immune responses [Kim and Maes, 2003]. The inhibition of Th1 derived interferon γ by Th2 cells is more complicated: the presence of IL-10 suppresses the synthesis of IL-12 by monocytes, macrophages and B cells. IL-12 is an important inducer of cell-mediated immunity and stimulates differentiation of CD4-helper T lymphocytes into T-helper cells that produce interferon γ. Stress promotes a shift in the balance of the reactions mediated by Th1 and Th2 cells [Elenkov and Chrousos, 1999; Kiecolt-Glaser *et al*., 2002].

The main immunosuppressive effects of cortisol include alterations in leukocyte traffic and function, a decrease in production of cytokines and mediators of inflammation and inhibition of their action on target tissues by the latter [Chrousos, 1995]. Glucocorticoids and catecholamines inhibit the production of IL-12 by antigen presenting cells such as monocytes, macrophages and dendritic cells without affecting the production of IL-10. IL-12 and TNF-α promotes Th1

responses and cellular immunity, whereas IL-10 suppresses both the production of IL-12 and the activity of Th1 cells and stimulates Th2 cell and humoral immune responses. The neuroendocrine mediators released by stress might thus cause a selective suppression of Th1 responses. Inhibition of Th1 but not Th2 cells explains the shift from the Th1 to Th2 mediated immune response, which impairs the cellular immune responses against various infections and some tumours that are normally mediated by the Th1 response (figure 3.5) [Agarwal and Marshall, 1998; Glaser *et al*., 1999; Elenkov and Chrousos, 1999; Kiecolt-Glaser *et al*., 2002; Vissoci Reichea *et al*., 2004].

Th2 derived IL-6, the main endocrine cytokine, plays a major role in the control of inflammation by stimulating glucocorticoid secretion and suppressing the secretion of TNF-α and IL-1 [Mastorakos *et al*., 1994]. In addition, catecholamines inhibit IL-12 and stimulate IL-10 secretion via β-adrenergic receptors, consequently causing suppression of innate and cellular immunity, and stimulation of humoral immunity.

Inhibition of innate immunity and T helper-1-related cytokines, such as interferon-γ and IL-12, and the stimulation of T helper-2-related cytokines, such as IL-10, is the result of a combination of the effects of glucocorticoids and catecholamines. A summary of the effect of stress on the immune system is illustrated in figure 3.5. Conditions, such as acute and chronic stress that contribute to a significant increase or decrease of local or systemic concentrations of these mediators via modulation of IL-12 and the balance between TNF-α and IL-10, might also play a role in the induction, expression and progression of some autoimmune and cardiovascular diseases, osteoporosis, rheumatoid arthritis, type 2 diabetes, allergic or atopic reactions and the growth of some tumours [Elenkov and Chrousos, 1999; Vissoci Reichea *et al*., 2004].

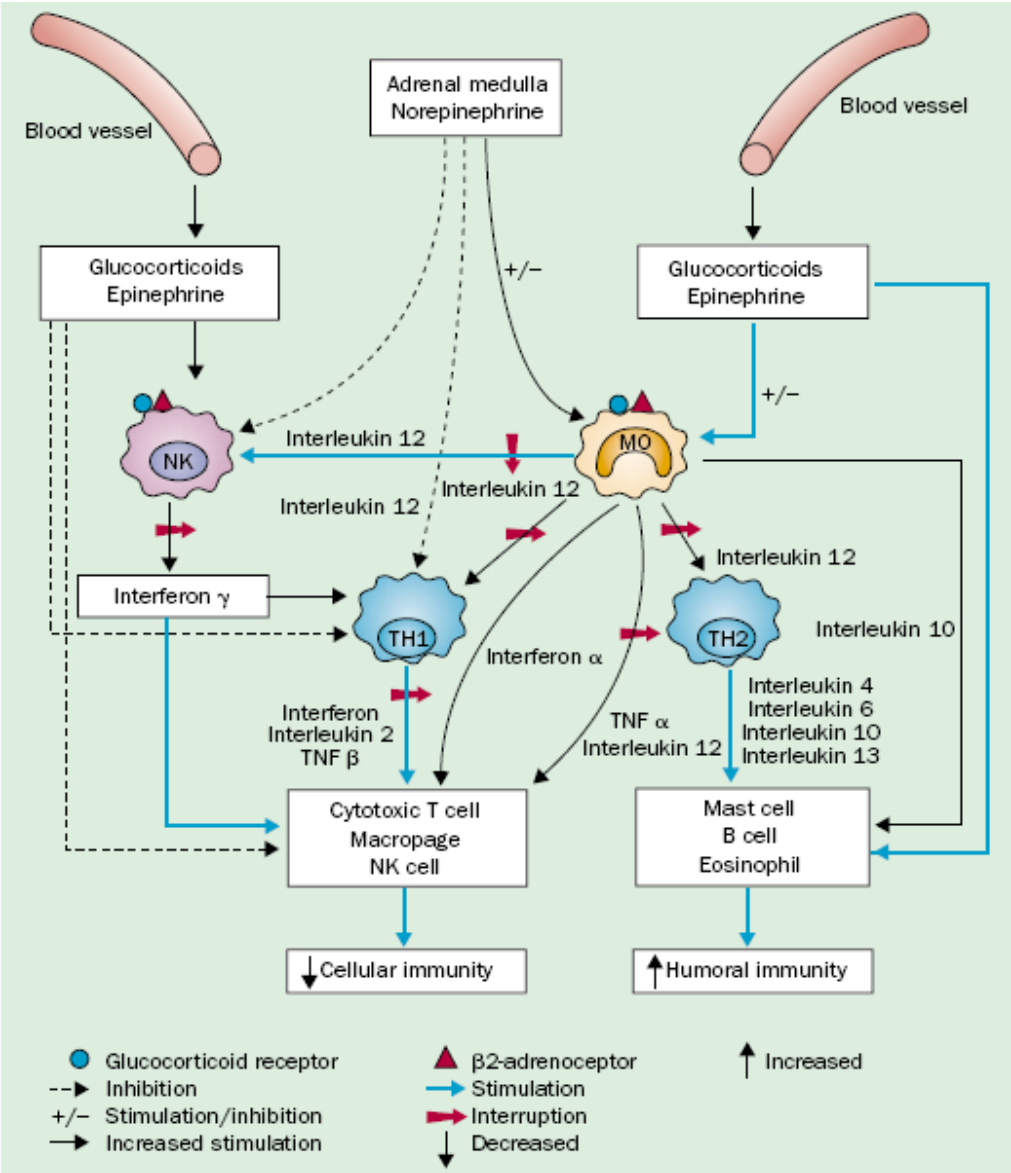


Figure 3.5. Systemic effects of the stress hormones glucocorticoids and catecholamines secreted by the adrenal gland and norepinephrine released by sympathetic nerve terminals. Solid lines indicated stimulation and dashed lines indicated inhibition. NK, natural-killer cells; MO, macrophage; Th1, T-helper lymphocyte type 1 cells; Th2, T-helper lymphocyte type 2 cells; TNF, tumour necrosis factor [Vissoci Reichea *et al.*, 2004].

3.4.2 Growth and development

Reproduction and growth are directly linked to the stress system and both are strongly inhibited by various components of the HPA axis. Suppression of the thyroid axis, probably through a direct action on thyrotropin secretion, is mediated by glucocorticoids. In a study conducted by Brtko *et al*. (2004), the effect of various stressors on the iodothyronine 5′-deiodinase (5´-DI) activity in rat liver was investigated. 5´-DI catalyzes the conversion of L-thyroxine (T4) to the active hormone, 3,5,3′-triiodothyronine (T3). Their results suggest that stress-induced elevation of plasma glucocorticoid levels significantly reduces the concentration of biologically active T3 by inhibiting 5´-DI activity [Brtko *et al*., 2004].

Inhibition of the growth axis occurs at various levels during stress. Long-term activation of the HPA axis, together with elevated cortisol levels, leads to suppression of growth hormone secretion and other growth factors [Magiakou *et al*., 1994].

Glucocorticoids have an inhibitory effect on gonadotropin-releasing hormone (GnRH) through an increase in somatostatinergic tone. Acting centrally, glucocorticoids also inhibit GnRH pulsatility, luteinizing hormone and follicle-stimulating hormone release [Saketos *et al*., 1993]. Increased glucocorticoid levels lead to target tissues becoming resistant to these hormones. In patients presenting with Cushing's syndrome, elevated plasma glucocorticoid levels suppress the pituitary-thyroid axis and pituitary-gonadal axis [Chrousos *et al*., 1998]. Cushing's disease is characterized by excessive cortisol levels, generally as a result of the hypersecretion of ACTH due to the presence of pituitary adenomas [Thomson and Craighead, 2008]. A reversible form of hypogonadotrophic hypogonadism is caused by cortisol which also directly inhibits Leydig cell function. Inhibition of gonadal function due to elevated cortisol levels in highly trained athletes, ballet dancers and individuals suffering from anorexia nervosa or starvation has been observed [Chrousos *et al*., 1998].

3.4.3 Glucose metabolism

Cortisol increases the concentration of blood glucose through its action on glycogen, protein and lipid metabolism. Chronic activation of the stress system increase visceral adiposity, decrease lean body (muscle and bone) mass and suppress osteoblastic activity (figure 3.6).

In the liver, cortisol stimulates hepatic gluconeogenesis, the conversion of non-carbohydrate sources, into glucose by increasing glycogen synthase and inhibiting the glycogen mobilizing enzyme, glycogen phosphorylase. The output of hepatic glucose is increased through the activation of key enzymes involved in gluconeogenesis, mainly glucose-6-phosphatase and phosphoenolpyruvate kinase (PEPCK) [Exton, 1979]. Glucose uptake and utilisation in peripheral tissues (muscle and fat) is inhibited by cortisol. Lipolysis is activated in adipose tissue, resulting in the release of free fatty acids into the circulation. Stimulation of hepatic lipoprotein synthesis leads to an increase in circulating cholesterol and triglycerides but HDL cholesterol levels decrease.

The permissive effect of glucocorticoids on other hormones, including catecholamines and glucagon, results in insulin resistance and an increase in blood glucose concentrations, at the expense of protein and lipid catabolism. In Cushing's syndrome patients, glucose intolerance frequently occurs and diabetes mellitus is present in about 30 % of patients.

Besides inducing insulin resistance in muscle tissue, cortisol also causes catabolic changes in muscle, skin and connective tissue. Epidermal cell division and DNA synthesis in skin and connective tissue is inhibited by cortisol with collagen synthesis and production being reduced. Glucocorticoids reduce muscle protein synthesis and causes muscle atrophy, specifically type II muscle fibers.

Glucocorticoid-induced osteoporosis is becoming a major health concern in Western populations with chronic glucocorticoid therapy being prescribed to 0.5 % to 1 % of the population. It is thought that the glucocorticoid excess observed in osteoporosis may result in the inhibition of osteoblast function which has been shown to be inhibited by cortisol [Canalis, 1996]. Intestinal

calcium absorption is also inhibited and renal calcium excretion is increased by glucocorticoids, thereby inducing a negative calcium balance in the body [Arlt and Stewart, 2005].

3.4.4 Blood pressure

Glucocorticoids increase the sensitivity of vascular smooth muscle to pressor agents such as catecholamines and angiotensin II while nitric oxide-mediated endothelial dilatation is reduced thus increasing blood pressure. In addition angiotensin biosynthesis is also increased by glucocorticoids. In the kidney, cortisol may cause sodium retention and potassium loss by acting on the distal nephron. Across the nephron, the glomerular filtration rate, proximal tubular epithelial sodium transport and free water clearance are increased by glucocorticoids [Arlt and Stewart, 2005].

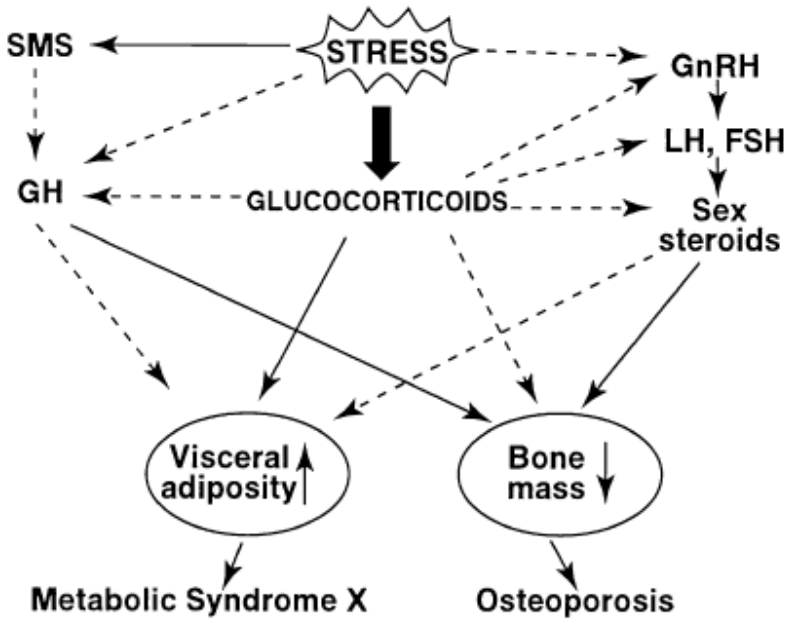


Figure 3.6. Detrimental effects of chronic stress on adipose tissue metabolism and bone mass. Solid lines indicate stimulation; dashed lines indicate inhibition [Tsigosa and Chrousos, 2002].

3.4.5. Psychiatric disorders

Emotional disorders such as depression and anxiety are a major cause of disability, impacting society both economically and socially [Kessler *et al*., 2005]. In spite of more than 50 years of research in drug discovery and development effective antidepressant therapies are as yet not available. There is thus a substantial medical need for effective drugs for the treatment of depressive illnesses [Fava, 2003]. A major obstacle in the development of more effective antidepressants is the lack of understanding of the full mechanisms underlying mood disorders.

An important target tissue for glucocorticoids is the brain as has been shown in clinical observations of patients presenting with depression, euphoria, psychosis, apathy and lethargy who have either glucocorticoid excess or deficiency. Clinical observations showed that the brain of patients who have glucocorticoid excess and deficiency is an important target tissue for glucocorticoids, with depression, euphoria, psychosis, apathy and lethargy being important symptoms. Both glucocorticoid and mineralocorticoid receptors are expressed in distinct regions in the rodent brain, including the hippocampus, hypothalamus, cerebellum and cortex [McEwen *et al*., 1986]. Neuronal death, particularly in the hippocampus, is caused by glucocorticoids and this may be the motivation of recent interest in the role of glucocorticoids in cognitive and memory function as well as neurodegenerative diseases such as Alzeihemer's disease. It has been demonstrated that the hormone, DHEA has neuroprotective effects in the hippocampus region [MacLusky *et al*., 2004; Hajszan *et al*., 2004].

CYP7B, the enzyme metabolizing DHEA to its 7α-hydroxylated metabolite, is highly expressed in the brain, particularly in the hippocampus. It has been shown in brain tissue of individuals with Alzheimer's disease that the expression of CYP7B is decreased significantly [Yau *et al*., 2003]. A characteristic shrinkage of the hippocampus region caused by glucocorticoid excess has been observed in patients who had presented with Cushing's syndrome. The question of whether the suppression of DHEA observed in adrenal Cushing's syndrome results in a more pronounced effect due to the loss of an apparent hippocampus protective factor remains to be elucidated.

Approximately 50 % of patients who have Cushing's syndrome endure psychiatric abnormalities. Agitated depression, lethargy, paranoia and overt psychosis are among the most common problems. Cognitive function and memory may also be affected and increased irritability is an early characteristic. Insomnia is common with both rapid eye movement and delta wave sleep patterns being reduced. The reduction of plasma cortisol by medical or surgical therapy results in a swift improvement in the psychiatric state of patients.

Melancholic depression or chronic anxiety disorder is a typical example of chronic hyperactivation of the stress response with hypersecretion of CRH and elevated cortisol levels being observed in these patients [Gold *et al*., 1988]. Melancholic depression may also be associated with conditions such as osteoporosis, features of metabolic syndrome, varying degrees of atherosclerosis, innate and Th1-directed immunosuppression, as well as certain infectious and neoplastic diseases due to the chronic elevation of cortisol [Chrousos, 2000]. Increased and prolonged HPA activation may also lead to other conditions such as anorexia nervosa, obsessive-compulsive disorder, panic anxiety, excessive exercise, irritable colon, hypertension, depression, chronic active alcoholism, diabetes mellitus types I and II, visceral obesity and hyperthyroidism [Tsigos *et al*., 1993].

The combination of chronic exposure to stressful events, especially during childhood, and genetic susceptibility factors play a key role in the development of a number of the psychiatric conditions mentioned above including major depressive disorder (MDD) and post-traumatic stress disorder. Following periods of chronic stress, the dysregulation of the negative feedback control of the HPA axis is implicated in the onset of these conditions [Schatzberg *et al*., 1985; Putignano *et al*., 2001; Marshall *et al*., 2002]. The association between depressive illness and abnormalities in HPA axis responsiveness has been the topic of many studies. Neuroendocrine irregularities including less pronounced diurnal cortisol rhythm, elevated serum and 24h urinary cortisol levels as well as adrenal gland hyperplasia have all been reported in severe depression. In addition, dysfunctional GR mediated negative feedback regulation of the HPA axis and changes in the AVP and CRF responsiveness of the HPA axis have also been observed. A deeper understanding of the excessive effects of cortisol on mood and cognitive function has been

gained by observing patients suffering from Cushings's disease [Thomson and Craighead, 2008]. A relationship between increased cortisol levels and its negative effects on cognition, verbal learning and other verbal functions has been suggested in a recent study [Roozendaal, 2002]. Successful restoration to the normal functioning of the HPA axis has often led to an improvement in the mood of patients suffering from Cushing's disease [Dorn *et al*., 1997].

A better understanding of the molecular and physiological mechanisms underlying depressive diseases could lead to a breakthrough in the improvement of therapies for these disorders. Evidence implicating a dysfunctional HPA axis in depressive illness is of great interest with the expectation that therapeutic agents that intervene with a hyperactive stress axis may lead to improved treatment therapies of these psychiatric disorders.

3.5 Summary

Hans Selye was the first to indicate that stress initiates a vital reaction in the body —- enhanced attention with the brain focused on the perceived threat. Aiding the body's response, cardiac output and respiration are accelerated, catabolism is increased and blood flow is redirected to provide the highest perfusion and fuel to the aroused brain, heart and muscles [Tsigos and Chrousos, 2002].

With the HPA axis being the final common pathway in the mediation of the stress response, the release of CRH in response to the stressor, followed by ACTH and the subsequent stimulation of cortisol production by the adrenal cortex is central to man's existence and survival. However, chronic activation of the HPA axis by elevated cortisol production has been shown to be detrimental to human health and has been linked to various clinical conditions.

Cortisol is the major stress hormone and mediates its effect on various organs and brain areas through the MR and the GR, both of which have a specific and selective distrubution in the brain and exhibit different affinites for cortisol. GRs are widely distrubuted in the brain and have been

found in regions such as the hippocampus, amygdala and the prefrontal cortex, which are relevant to cognition [Bao *et al*., 2008].

Hypersecretion of adrenal glucocorticoids leading to a sustained activation of the central and peripheral sympathetic systems and the desensitisation of the glucocorticoid receptors result in a decreased sensitivity to feedback inhibition by cortisol. The function of the normal stress response lies in the maintainance of stability or homeostasis, with long-term or chronic activation of the stress system resulting in clinical conditions characterised by an increased risk of obesity, heart disease, insulin resistance, metabolic syndrome, hypertension, osteoporosis, depression and a variety of other illnesses, some of which could lead to death.

Cortisol is synthesized in the adrenal cortex and the enzymes involved in the biosynthesis of cortisol in the adrenal gland is discussed in the following chapter.

Chapter 4

Adrenal steroidogenic cytochrome P450 enzymes

4.1 Introduction

The cytochrome P450 (P450) enzymes represent the largest superfamily of heme-containing proteins found in bacteria, fungi, plants and animals [Nelson *et al*., 1996]. The discovery of P450 enzymes began in 1955 when Williams and Klingenberg observed a pigment with an unusual carbon monoxide-binding spectrum in rat liver microsomes. The appearance of a broad but intense absorption band at 450 nm was obtained upon bubbling carbon monoxide into a NADH-reduced liver microsomal suspension. Klingenberg was unable to determine the chemical nature of the pigment and no new findings were reported until 1962 when Omura and Sato presented conclusive spectral evidence for its hemoprotein nature. They proposed the name "P450" indicative of a pigment with an absorption at 450 nm [Omura *et al*., 1964]. The term cytochrome is, however, unsuitable since P450 enzymes generally act as oxygenases rather than just electron carriers like other cytochromes (cytochrome a, b c) [Nelson *et al*., 1996].

These enzymes share a common structural topology with a heme moiety central to the active site and are involved in the metabolism of mostly small hydrophobic molecules. The P450 enzyme family metabolises a variety of endogenous compounds, participate in the activation or deactivation of many carcinogens and detoxify various xenobiotics. In humans, P450 enzymes metabolise numerous drugs and therefore are of great interest to pharmacologists and toxicologists. These features make P450 enzymes the most versatile of biological catalysts.

The steroidogenic P450 enzymes are fairly specific in their choice of substrates but those located in the hepatic endoplasmic reticulum catalyse a surprisingly large number of chemical reactions with an almost unlimited number of biological and xenobiotic compounds that serve as substrates. Some examples of xenobiotics that can act as substrates for P450 enzymes are: drugs (including antibiotics), procarcinogens, antioxidants, organic solvents, anesthetics, dyes,

pesticides, alcohols, odorants, and flavourants, and a variety of unusual substances in plants and microorganisms, which, despite their biological occurrence, are foreign to animals and humans [Porter *et al*., 1991]. Physiologically important substrates include steroids, eiconosanoids, fatty acids, lipid hydroperoxides, retinoids, acetone and acetol [Coon *et al*., 1992].

4.2 Enzymology/mechanism of action of P450 systems

P450 enzymes are made up of ~ 400 to 500 amino acids and contain a single heme prosthetic group. The iron protoporphyrin IX complex is bound, in part, by hydrophobic forces in the active site of P450 enzymes (figure 4.1). The specific function of hemoproteins is determined mainly by the axial heme iron ligands. Contrary to the oxygen-binding proteins (myoglobin, hemoglobin) which have a histidine residue as the fifth ligand, the P450 enzymes have a thiolate anion provided by a cysteine residue. The characteristic thiolate ligand plays a role in the specific function of P450 enzymes as well as the unusual spectral and catalytic properties of these enzymes. It is believed that the sixth coordinate position may be occupied by an exchangeable water molecule and when the iron is reduced, oxygen can be bound in the sixth position [Omura and Sato, 1964; White and Coon, 1980; Ruckpaul and Rein, 1989; Segall *et al*., 1998].

The reaction cycle of P450 enzymes has been a topic of investigation for many years [Coon, 2005]. All P450 enzymes share a common reaction cycle both in regard to the spin- and coordination state of the heme iron and their ability to undergo reduction, regardless of their phylogenetic origin or cellular localization. The first enzyme that was accessible for purification and agreeable for a variety of biochemical and biophysical studies was P450cam (CYP101). CYP101 is a soluble protein found in the bacterium *Pseudomonas putida* that oxidizes camphor. P450cam was the first P450 to be crystallized and the X-ray data on this enzyme has served as a basis for structural insights into the structure-function relationships of P450 enzymes [Narasimhulu, 2007; Poulos *et al*., 1986].

The catalytic reaction takes place on Fe^{3+} in the heme moiety found at the active site of all P450 enzymes. Fe^{3+} (Ferric iron) can be either in the low spin state (S = ½), in which the five 3d

electrons are maximally paired, or high spin state (S = 5/2) in which the five 3d electrons are maximally unpaired. Six-fold coordinated Fe^{3+} is usually found to be low spin whereas five-fold coordinated Fe^{3+} is found in the high spin state. The nature of the axial heme ligands thus has an important effect on the spin state of the P450 enzymes.

In its resting, subtrate-free state, the heme iron (Fe^{3+}) is in a low spin state. A water molecule forms the sixth axial ligand of Fe^{3+} in the substrate-free form, thereby stabilizing the low spin state of the ion. The substrate-free P450 exhibition of a Soret absorption maximum at 417 - 420 nm is associated with the low spin state of the Fe^{3+} [Segall *et al*., 1998].

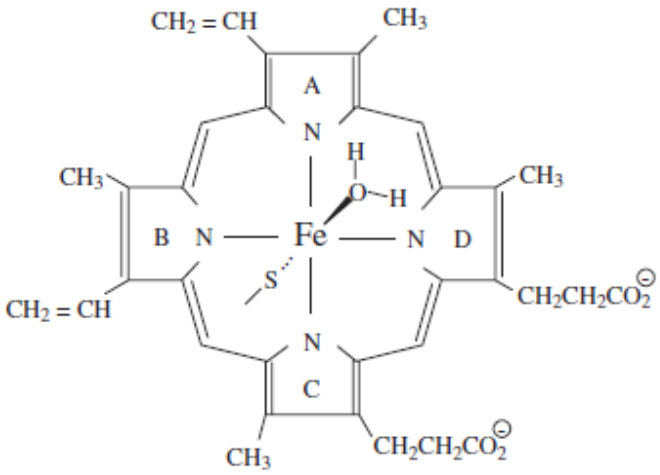


Figure 4.1. P450 heme moiety structure displaying the distal (water) and proximal (thiolate) ligands present in the resting state of the enzyme with the prophyrin ring system consisting of the four pyrrole groups (reproduced from Lewis et al., 2006).

The catalytic cycle of the P450 enzyme consists of various steps that are involved in the overall process:

1) In the first step of the catalytic reaction the substrate binds to the ferric P450 enzyme, disturbing the spin state equilibrium of the enzyme, changing the state of the heme iron from a low spin to that of a high spin. X-ray crystallography of substrate-bound complexes of CYP101 shows that the iron-ligated water molecule at position sixth is displaced. This results in the change of the Fe^{3+} from a six-fold to a five-fold coordination state with the Fe^{3+} moving out of the plane of the heme. Characteristic spectral changes of the heme chromophore in the near UV

spectral region (between 300 and 400 nm), the Soret region (between 400 and 500 nm) and in the visible spectral region (500 to 780 nm) are observed upon binding of a substrate to cytochrome P450. An apparent binding constant (K'_s) can be calculated from the substrate-induced spectral changes.

The majority of substrates metabolized by P450 enzymes produce a Type I difference spectrum on binding, characterized by a Soret absorption minimum at ≈ 420 nm and a maximum between 385-390 nm [Ruckpaul and Rein, 1989]. This is indicative of a change in spin state of the ferric iron from low spin to high spin. The change in spin state is accompanied by a change in redox potential of the heme Fe^{3+} which makes the reduction of the P450 enzyme energetically favorable and allows the catalytic reaction to proceed [Segall *et al*., 1998].

Certain compounds, some of which have a hydroxyl (OH) funcional group such as alcohols, ketones and certain drugs, produce a reverse type I spectrum, with an absorption maximum between 409 - 445 nm and a minimum at 365-410 nm, upon binding to P450 enzymes. Although these compounds bind to a site other than the heme group they also displace the water molecule due to the lipophyllic interaction in the active site. A number of compounds also produce a type II difference spectrum, with an absorption maximum between 425 - 435 nm and minimum between 390 - 405 nm. Many of these type II compounds, the majority of which are inhibitors, interact directly with the heme by binding at the sixth coordination position and displacing one of the axial ligands from the heme iron [Ruckpaul and Rein, 1989; Omura *et al*., 1993].

2) The change in spin state of the P450 enzyme facilitates the uptake of the first electron via the electron transfer chain and the ferric heme iron is reduced to the ferrous state.

3) The release of water as the sixth axial ligand allows molecular oxygen to bind to the ferrous P450 enzyme to initiate the oxidative reactions. The binding of the dioxygen molecule converts the ferrous iron to a low spin state. The binding of molecular oxygen is stabilized by the electron delocalization/distribution between sulphur, iron and dioxygen molecule with the formation of a ferric superoxide specie.

4) The second electron is transferred via the cytochrome P450 redox partner, resulting in the activation of the oxygen and the reinstatement of the iron to its ferric form in the highly reactive Fe^{3+}-O_2^- species.

5) The next step involves the splitting of the oxygen-oxygen bond with the generation of an "activated oxygen", possibly an iron-oxene species, and the release of H_2O from the active centre. Hydrogen peroxide may form with the possible uptake of two protons by the reactive Fe^{3+}-O_2^- species.

6) Finally, characteristic of the monoxygenase reaction, the activated oxygen atom may react with the substrate molecule located in close proximity to the activated oxygen atom.

7) The oxygenated product may also be released from the P450 enzyme resulting in the restoration of the P450 to the resting ferric state [Coon, 2005; Omura *et al*., 1993]. The various steps involved in the hydroxylation reaction of the P450 enzymes are illustrated in figure 4.2.

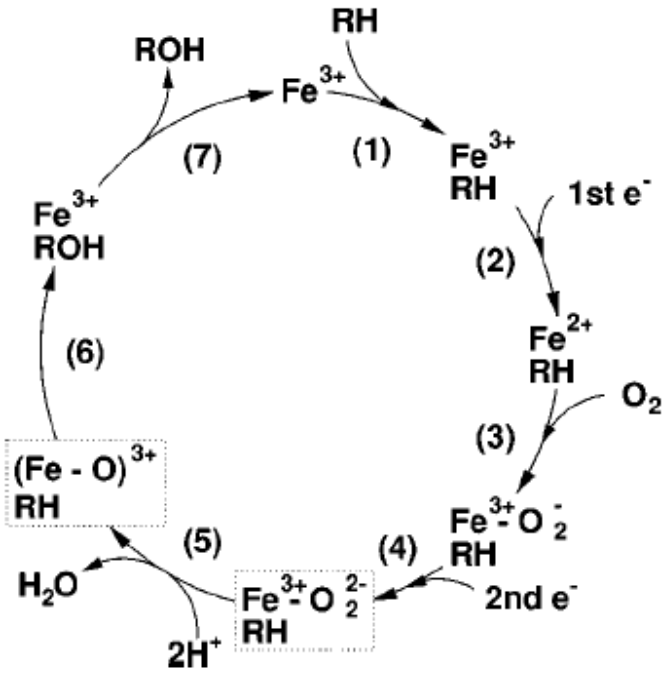


Figure 4.2. Overview of the catalytic cycle of cytochrome P-450 enzymes, RH represents the substrate and ROH the product [reproduced from Segall *et al*., 1998].

4.3 Redox partners

The rate of reactions catalysed by P450 enzymes is largely dependent on the rate of electron transfer from its redox partners. The majority of reactions start with the transfer of electrons from NAD(P)H. Hydroxylation, epoxidation, peroxygenation, deamination, desulfuration, dehalogenation as well as reduction reactions, have all been demonstrated. There has been no evidence showing that charge-charge interactions contribute to the binding by the cytochrome as most of the substrates are lipophyllic. The transformation of some substrates is essential for life, such as the conversion of cholesterol to corticoid and sex hormones, metabolism of drugs, fatty acids, bile acids, vitamin D and steroid hormones. P450 enzymes are also involved in the detoxification of carcinogens and xenobiotics, which leads to the formation of more polar compounds that are more readily excreted directly of after conjugation with water-soluble agents such as glucuronic acid and glutathione [Miller, 2005].

During the biosynthesis of steroid hormones, the hydroxylation and cleavage of the steroid substrates are catalyzed by the P450 enzymes. The general stoichiometry of the hydroxylation reaction, where RH represents the substrate and ROH the product, is:

$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$

where cytochrome P450 acts as a monooxygenase in the reaction, utilizing reduced NADPH as the electron donor for the reduction of molecular oxygen. The P450 enzyme activates the oxygen and one oxygen atom is inserted into the substrate as a hydroxyl group (ROH), with the reduction of the other atom to water [Coon, 2005].

P450 enzymes are characterized by highly negative oxidation-reduction potentials without distinct differences between isozymes and species. This redox potential is related to the presence of both the thiolate ligand and the local nonpolar heme environment. A shift of the negative redox potential to a more positive value is observed upon substrate binding which favors the uptake of an electron [Ruckpaul and Rein, 1989].

Two distinctive electron transfer systems are involved in the transfer of electrons from NADPH to the substrate. The two classes of P450 enzymes involved in steroid hormone biosynthesis are membrane-bound proteins and they are classified based on the electron transfer system they utilize — class I enzymes are located in the mitochondria: cytochrome P450 side chain cleavage (CYP11A), cytochrome 11β-hydroxylase (CYP11B1) and aldosterone synthase (C11B2), and class II are located in the endoplasmic reticulum (microsomal region): cytochrome 17α-hydroxylase/17,20 lyase (CYP17), aromatase (CYP19) and cytochrome 21-hydroxylase (CYP21) [Nelson *et al*., 1996].

In the mitochondrial system, two proteins are involved in the transfer of electrons — adrenodoxin reductase (ADXR), a FAD containing flavoprotein, and adrenodoxin (ADX), a ferrodoxin type iron sulfur protein. A high potential electron is transferred from NADPH to ADXR, and then sequentially to ADX, P450 and finally the substrate as shown in figure 4.3A. Only one redox protein is involved in the microsomal system, cytochrome P450 oxidoreductase (POR), a protein that contains two flavins, FAD and FMN (figure 4.3B). Electrons are

transferred from NADPH to FAD and then sequentially to FMN, P450 and the substrate (figure 4.3B) [Hanukoglu, 1992]. Some microsomal P450 enzymes may also accept a second electron from cytochrome b5 [Kominami *et al*., 1992].

Cytochrome b5 is an allosteric regulator that selectively enhances the 17,20-lyase activity of CYP17. The increased intracellular expression of cytochrome b5 in the gonads accounts for the preferential production of androgens by these tissues. Cytochrome b5 expression in the adrenal is not very high and the streroidogenesis pathway in the adrenal favours the synthesis of glucocorticoids and mineralocorticoids [Wagner *et al*., 2008, Dharia *et al*., 2004].

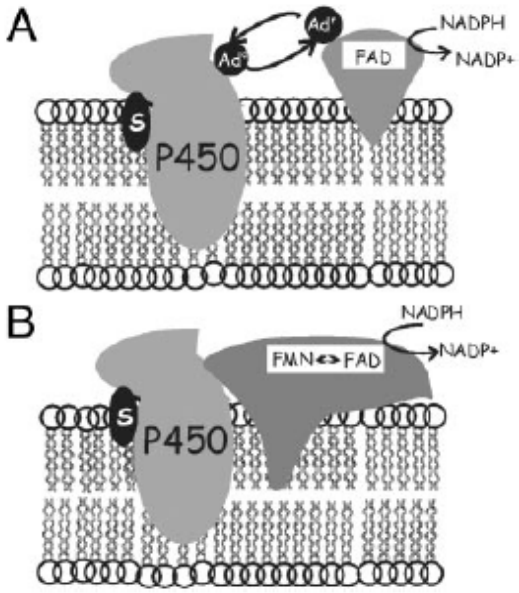


Figure 4.3. Schematic representation of the mitochondrial electron transfer system (A) and microsomal electron transfer system (B) [reproduced from Payne and Hales, 2004].

4.4 Steroid hormone biosynthesis in the adrenal gland

Adrenal steroid hormones play a role in the regulation of the water and electrolyte balance, in the metabolism of carbohydrates, lipids and proteins as well as in sexual development and maintenance of sexual characteristics. The P450 enzymes make up one of the two major classes of enzymes involved in the biosynthesis of adrenal steroid hormones, corticosterone, cortisol and

aldosterone. The other class of enzymes involved is the hydroxysteroid dehydrogenases. The hydroxysteroid dehydrogenases are involved in the reduction and oxidation of steroid hormones requiring NAD/NADP as acceptors and their reduced forms as donors of reducing equivalents. A difference between the P450 enzymes and the hydroxysteroid dehydrogenases is that each of the P450 enzymes is a product of a single gene whereas the several isoforms for the 3β HSDs and several isozymes of the 17β-hydroxysteroid dehydrogenase (17βHSDs) are each a product of a distinct gene [Payne and Hales, 2004].

Adrenal steroid hormone biosynthesis pathways are well established with the molecular identities and zonal expression of the responsible steroidogenic enzymes that have been revealed. The adrenal cortex consists out of three functional regions: the outer zona glomerulosa, the middle zona fasciculata and the inner zona reticularis (figure 4.4) [Messiano and Jaffe, 1997]. The zona glomerulosa produces aldosterone under control of the renin-angiotensin system. The zona fasciculata secretes glucocorticoids under the regulation of ACTH, whereas the zona reticularis in humans secretes dehydroepiandrosterone (DHEA) and its sulfated derivative in response to ACTH [Hammer *et al*., 2005]. Adrenal P450 enzymes exhibit zone specific expression, important for catalyzing specific reactions in the steroidogenic pathway. For example, CYP11A1 the enzyme catalysing the first step in the synthesis of all steroid hormones, is expressed in all 3 zones. CYP17 is an important enzyme at the branch point of steroidogensis channelling steroid intermediates to either the glucocorticoid or adrenal androgen pathways. It is expressed in both the zona fasciculate where it hydroxylates pregnenolone (PREG) and progesterone (PROG) and in the zona reticularis where the lyase activity channels the intermediates towards androgen production. CYP21 is located in the zona glomerulosa and fasciculata only and thus channels intermediates into the mineralocorticoid and glucocorticoid pathways, respectively. CYP11B1 and CYP11B2 are both expressed in the zona glomerulosa with CYP11B2 catalysing the biosynthesis of mineralocorticoids in the zona glomerulosa with CYP11B1, also expressed in the zona fasciculata and reticularis, catalysing the biosynthesis of glucocorticods, cortisol and corticosterone [Ghayee and Auchus, 2007].

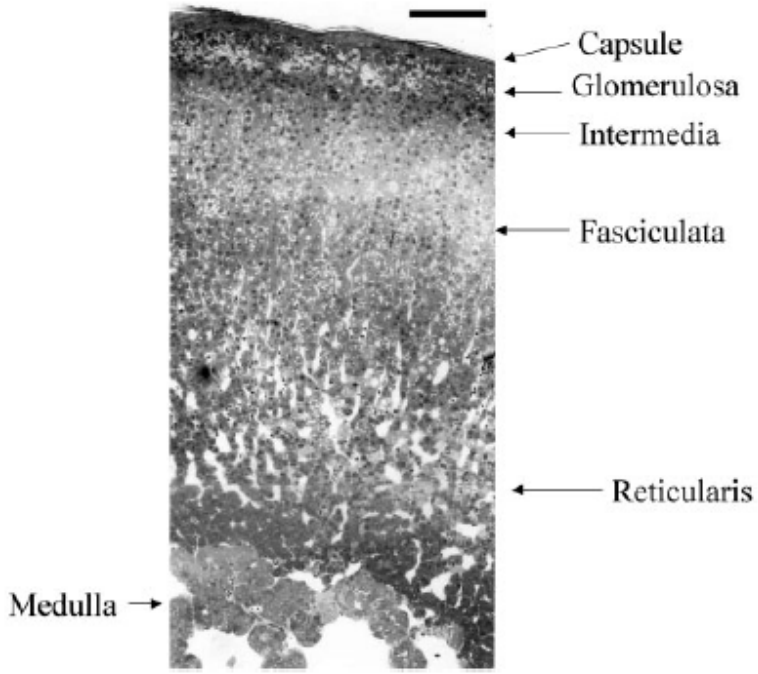


Figure 4.4 Zonal arrangement of adrenal gland [reproduced from Vinson, 2003].

Cholesterol, the precursor from which all endogenous steroids are derived, enters the cell as a lipoprotein-bound molecule and is stored in cytoplasmic vacuoles, inserted into the outer mitochondrial membrane. Upon release from the pituitary gland, ACTH binds to its receptor, melanocortin receptor type 2 (MC2R), on adrenal cells which subsequently leads to the mobilizing of cholesterol that is made available for steroid biosynthesis.

Cholesterol is transferred from the outer to the inner mitochondrial membrane where the cholesterol side chain cleavage enzyme (CYP11A1) is located and steroidogenesis begins with the cleavage of the side chain being catalysed by this enzyme. This initial reaction, in which cholesterol is converted to PREG, is tightly regulated and allows rapid production of steroids in response to stimuli. In humans, only certain cells have the ability to convert cholesterol to PREG: testicular Leydig cells, placental trophoblast cells, ovarian thecal cells, corpus luteum cells, certain neuronal cells in the brain and of course adrenal cortical cells. The steroidogenic acute regulatory protein (StAR) is a key protein involved in the transfer of cholesterol from the outer to the inner mitochondrial membrane [Clark *et al*. 1994; Ghayee and Auchus, 2007; Guo *et al*., 2003]. StAR is capable of moving a significant number of cholesterol molecules before it

becomes inactive and thus appears able to serve either a transfer or a catalytic role [Artemenko *et al*. 2001].

After the conversion of cholesterol to PREG by the mitochondrial side-chain cleavage system, PREG may be diverted toward one of three different pathways. These pathways are compartmentalized and occur in a specific region in the adrenal cortex.

First, PREG may proceed down the mineralocorticoid pathway toward aldosterone in the zona glomerulosa. In this pathway, PREG is converted to PROG by the 3β-HSD enzyme, PROG is converted by CYP21 to deoxycorticosterone which is subsequently converted to corticosterone and aldosterone by CYP11B2 (figure 4.5).

Second, PREG can be 17α-hydroxylated to 17OH-PREG by CYP17 after which 3β-HSD converts it to 17OH-PROG. PROG may also be 17α-hydroxylated to 17OH-PROG by CYP17. CYP21 is responsible for converting 17OH-PROG to deoxycortisol and this reaction occurs in the endoplasmic reticulum of the zonae fasciculata and reticularis. The intermediate is transferred to the mitochondria where CYP11B1 catalyses the conversion of deoxycortisol to the principal glucocorticoid, cortisol.

Third, after the hydroxylation at C17, the C17-20 bond of 17OH-PREG and 17OH-PROG interemediates may be cleaved by the same enzyme, CYP17 yielding the C19,17-ketosteroids, DHEA and androstendione. DHEA may also be converted to androstenedione by 3β-HSD. These adrenal androgens are synthesised in the zona reticularis. Androstenedione may be subsequently converted to sex steroids [Miller and Auchus, 1997]. The steroidogenic enzymes involved in these pathways are discussed in the following section.

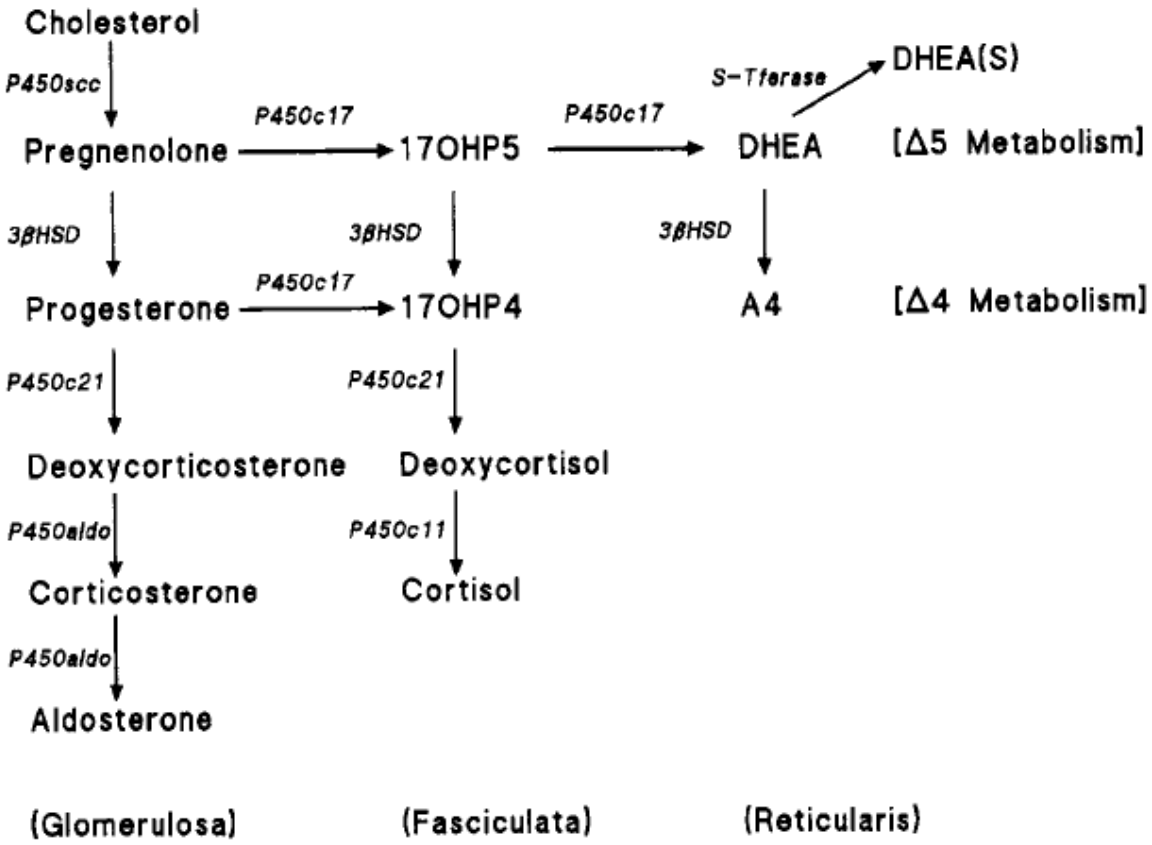


Figure 4.5. Adrenal steroidogenesis, P450scc, cytochrome P450 side chain cleavage; P450c17, cytochrome 17α-hydroxylase/17,20 lyase; 3βHSD, 3β-hydroxysteriod dehydrogenase; P450c21, cytochrome 21-hydroxylase; P450aldo, aldosterone synthase; P450c11, cytochrome 11β-hydroxylase; S-Tferase, sulfotransferase [reproduced from Conley and Bird, 1997].

4.4.1 Cytochrome P450 side chain cleavage

The first and putative rate-limiting step in steroid hormone biosynthesis is catalyzed by CYP11A1 with the conversion of cholesterol to PREG. Cholesterol is the common precursor for all steroid hormones and is stored by most steroid hormone-producing cells in the form of cholesterol esters within cytosolic lipid droplets. Several distinct phases of cholesterol transport to CYP11A1 exists, the first being receptor-mediated endocytosis of low density lipoprotein (or high density as in rodents) from the plasma to lipid droplets. The next step involves the hydrolysis of cholesterol esters followed by the transfer of cholesterol to mitochondria and

intramitochondrial transfer of cholesterol to CYP11A1. Phases one and two are regulated by ACTH via the stimulation of LDL receptors and cAMP-dependent protein kinase activation of cholesterol esterases respectively [Capponi, 2002; Privalle *et al*., 1983].

Three sequential oxidation reactions of cholesterol are catalyzed by CYP11A1, each requiring one molecule of oxygen and NADPH (figure 4.6). During the first reaction, hydroxylation occurs at C22 followed by the hydroxylation at C20 to yield 20,22-hydroxycholesterol which is subsequently cleaved between C20 and C22 to yield the C21 steroid PREG [Burstein and Gut, 1976; Payne and Hales, 2004]. CYP11A1 is expressed in all three zones of the adrenal cortex.

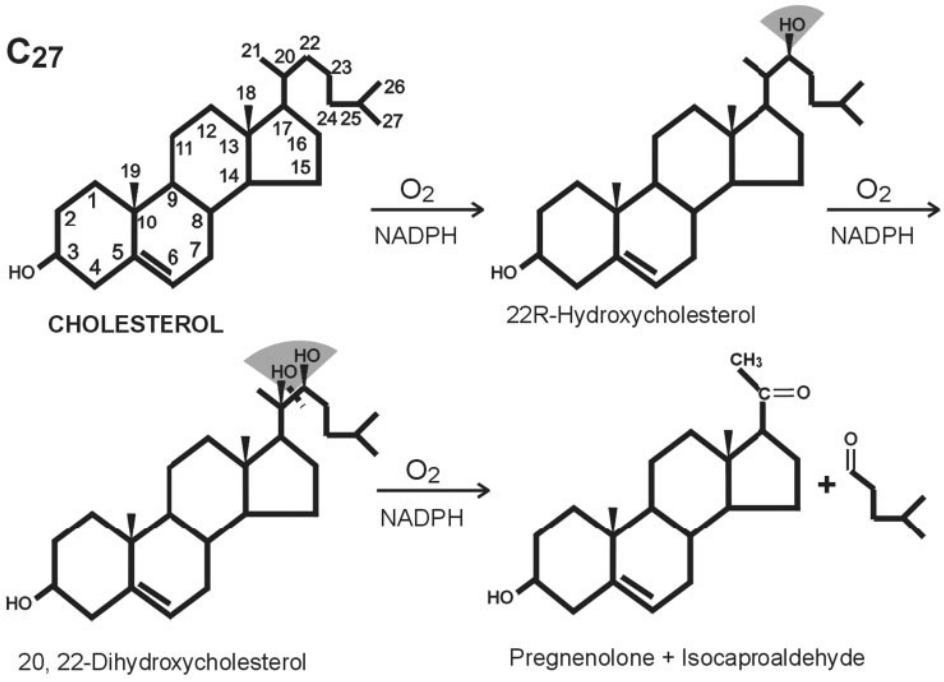


Figure 4.6. Enzymatic reactions catalyzed by CYP11A1 [reproduced from Payne and Hales, 2004].

4.4.2 Cytochrome 17α-hydroxylase/17,20 lyase

Two mixed function oxidase reactions are catalyzed by CYP17 utilising the microsomal electron transfer system, POR — the 17α-hydroxylation of the C21 steroids, PREG, a Δ^5 steroid and PROG, a Δ^4 steroid, followed by the cleavage of the C17-20 bond to produce the C19 steroids,

DHEA or androstenedione, respectively (figure 4.7). One molecule of NADPH and one molecule molecular oxygen is required for each reaction and 17α-hydroxypregnenolone (17OH-PREG) or 17α-hydroxyprogesterone (17OH-PROG) is formed as intermediates in this two-step reaction. It was first believed that two enzymes were responsible for catalyzing each reaction. However, studies showed that a single purified protein catalyzed both the17α-hydroxylation and the C17-20 cleavage (lyase activity). Subsequent cloning and expression of bovine CYP17 cDNA further confirmed that both reactions are catalyzed by CYP17 which is encoded by a single gene [Zuber *et al*., 1986].

The absence of CYP17 expression in the human adrenal zona glomerulosa directs steroidogenesis to the minerlocorticoid, aldosterone. The 17α-hydroxylase activity of CYP17 in the zona fasciculata exeeds the 17,20 lyase activity and this results in cortisol production. The high 17,20-lyase activity in the zona reticularis results in the consequent synthesis of the C19 precursors of sex steroids, mainly DHEA and its sulfate, along with androstenedione [Miller *et al*., 1997]. Sex steroid production may be driven by CYP17 with the conversion of PREG to 17OH-PREG and then to DHEA along the Δ^5 pathway. DHEA is subsequently converted to androstenediol by 17βHSD3, and testosterone biosynthesis is completed by 3βHSD2. On the other hand, flux may simultaneously occur via the Δ^4 pathway with PROG being converted to 17OH-PROG and subsequently to androstenedione [Flück *et al*., 2003]. Although both the hydroxylation and lyase reactions are catalyzed by CYP17 in various species, the dominant pathway synthesizing C19 steroids varies and is species specific, mainly due to species-dependant changes observed in 17,20-lyase activity in the Δ^4 and Δ^5 pathways. Human and bovine enzymes preferentially catalyse the Δ^5 pathway, whereas the Δ^4 pathway is the preferred pathway in rodents [Brock and Waterman, 1999]. Cytochrome b5 favors 17,20-lyase activity allosterically and is preferentially expressed in the zona reticularis of the adrenal, where the C19-steroids are produced [Auchus *et al*., 1998; Mapes *et al*., 1999]. The lyase activity of rat CYP17 with 17OH-PROG as substrate is also stimulated by cytochrome b5 but the fold increase is small in comparison to the increase observed with human CYP17 [Brock and Waterman, 1999].

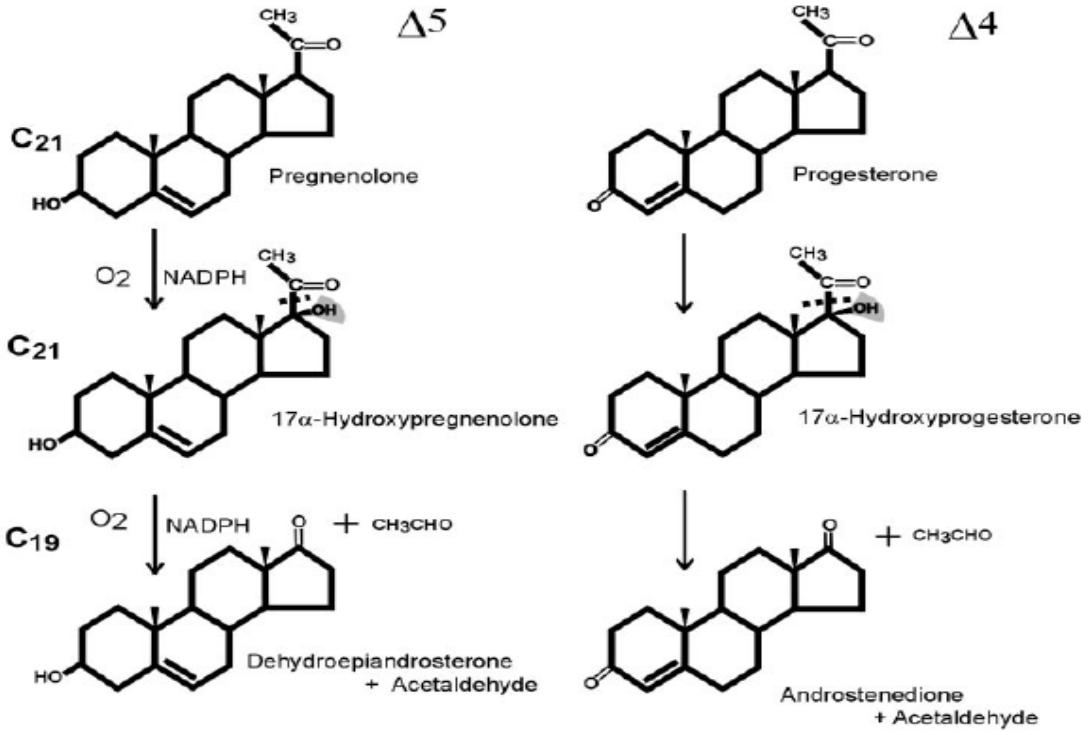


Figure 4.7. Enzymatic reactions catalyzed by CYP17 [reproduced from Payne and Hales, 2004].

4.4.3 3β-Hydroxysteriod dehydrogenase

The 3-βHSD enzyme is a membrane-bound enzyme that may be distributed to both the mitochondrial and microsomal membranes, depending on the type of cell in which they are expressed. The enzyme catalyses the conversion of the 3β-hydroxy-5-ene steroids into 3-keto-4-ene steroids. Unlike the steroidogenic P450 enzymes, each of which is encoded by a single gene in the human, rat and mouse genomes, there are at least 2-3 homologous genes encoding the 3-βHSD. Many isoforms of this enzyme have been isolated and characterized in the past decade. In humans, two distinct isoforms have been identified, 3-βHSD I and 3-βHSD II, both of which function as steroid dehydrogenase/isomerases. The two isoforms found in humans and rats can utilize either PREG, 17OH-PREG or DHEA as substrates converting them to the Δ^4-3-ketosteroids PROG, 17OH-PROG and androstenedione respectively (figure 4.8) [Payne and Hales, 2004, Labrie *et al*., 1992].

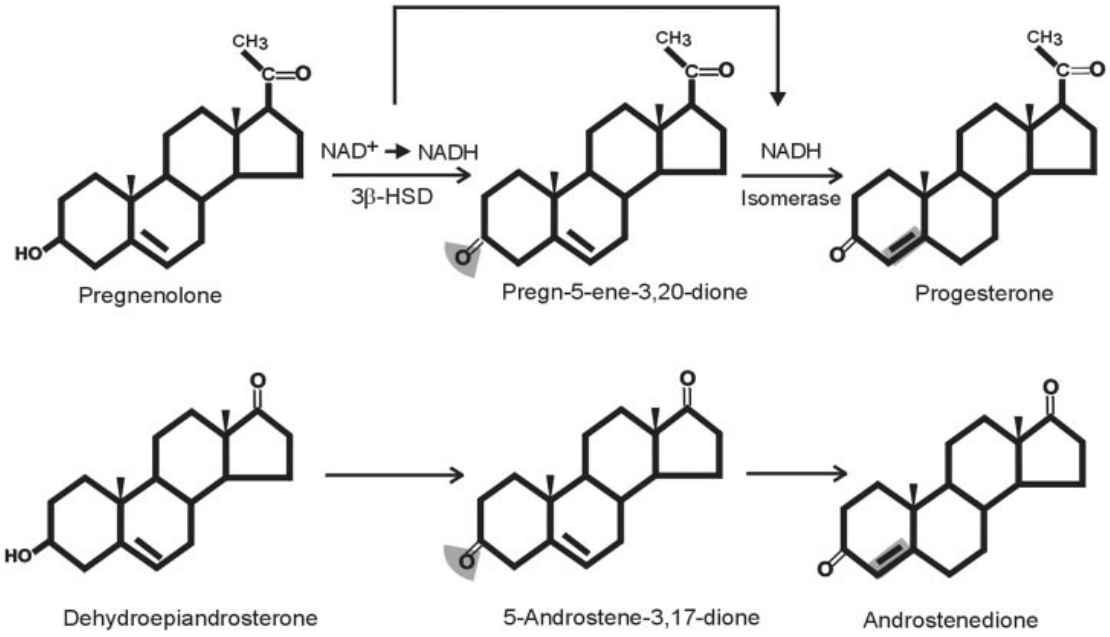


Figure 4.8. Enzymatic reactions catalyzed by 3-βHSD [reproduced from Payne and Hales, 2004].

4.4.4 Cytochrome 21-hydroxylase

CYP21 expression is exclusive to the adrenal cortex, catalyzing the reactions that direct the steroidogenic pathway toward the production of glucocorticoids, cortisol and corticosterone and the mineralocorticoid, aldosterone — the hydroxylation at C21 of PROG and 17OH-PROG to yield 11-deoxycorticosterone (DOC) and 11-deoxycortisol respectively (figure 4.9) [Crawford *et al*., 1992]. All three zones of the adrenal cortex, the zona reticularis, zona fasciculata and zona glomerulosa, express CYP21 [Parker *et al*., 1985]. A deficiency in CYP21 hydroxylation results in gluco- and mineralocorticoid shortage due to the shunting of the steroid biosynthesis pathway in the direction of androgen production [White and New, 1992].

In bovine and guinea pig microsomes and reconstituted systems, CYP21 has a higher activity for 17OH-PROG than for PROG [Higuchi *et al*., 1991; Kominami *et al*., 1980]. It was found that there was no significance in the difference in the rate of substrate binding to CYP21 in liposomal membranes between the two substrates [Kominami *et al*., 1986]. The analyses of kinetic data

revealed that the rate-determining step in 21-hydroxylation of PROG in the steady state was the dissociation of product from CYP21, whereas the conversion to deoxycortisol was the rate-determining step in the reaction of 17OH-PROG [Kominami *et al*., 1996].

P450 enzymes with steroid 21-hydroxylation activity have been found in other tissues e.g liver but show little similarity to the steroidogenic CYP21 as they represent products from other genes [Tukey *et al*., 1985]. Although humans have two CYP21 genes, CYP21 A and B, which are duplicated in tandem with the C4A and C4B genes, only the CYP21B gene encodes an active enzyme [Morel *et al*., 1989; Chaplin *et al*., 1986]. An eight-base deletion, a one-base insertion and a transition mutation in the human CYP21A gene is most likely the reason why premature termination of translation occurs [White *et al*., 1986; Higashi *et al*., 1986].

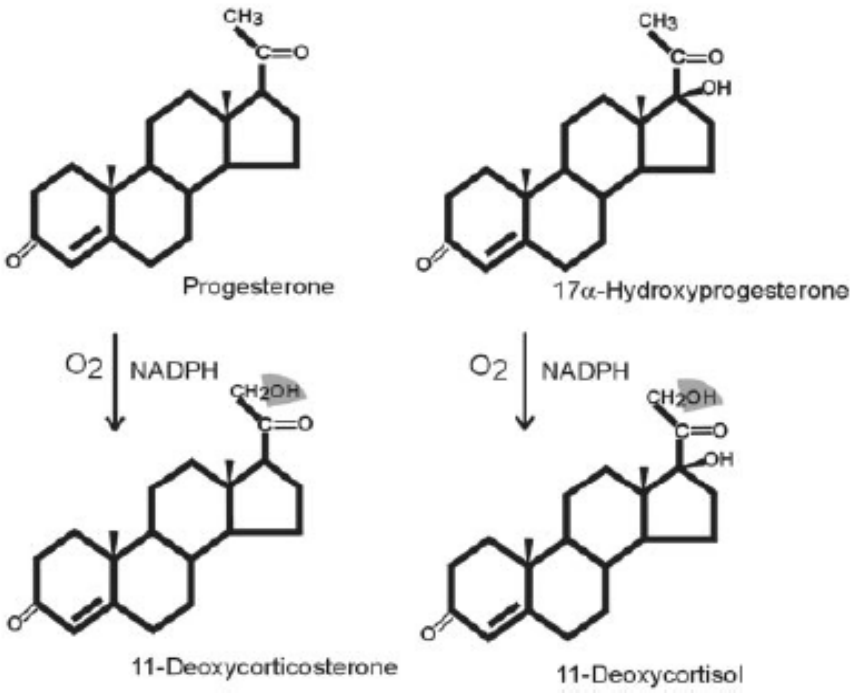


Figure 4.9. Enzymatic reactions catalyzed by CYP21 [reproduced from Payne and Hales, 2004].

4.4.5 Cytochrome 11β-hydroxylase and aldosterone synthase

Two reactions are catalyzed by CYP11B1 in the steroidogenesis pathway: the 11β-hydroxylation of 11-deoxycortisol and 11-deoxycorticosterone to yield cortisol and corticosterone respectively

(figure 4.10). The conversion of 11-deoxycorticosterone to aldosterone is catalyzed by CYP11B2 in three sequential reactions: the 11β-hydroxylation of 11-deoxycorticosterone, the hydroxylation of C18, followed by oxidation of the C18 hydroxyl group to yield an aldehyde group at that position (figure 4.11). Each of these reactions requires one molecule of NADPH and oxygen [Kawamoto *et al*., 1992; Pane and Hales, 2004].

Evidence has been provided that corticosterone is not used effectively by CYP11B2 as a substrate for aldosterone biosynthesis. CYP11B2 only catalyses the conversion of 11-deoxycorticosterone, with no involvement of CYP11B1, in the biosynthesis of aldosterone [Mellon *et al*., 1995; Payne and Hales, 2004]. The main sites of CYP11B1 expression are in the adrenal zonae fasciculata and reticularis with some expression in the mitochondria of the zona glomerulosa. In contrast, expression of CYP11B2 is restricted to the zona glomerulosa [Ogishima *et al*., 1991; Payne and Hales, 2004].

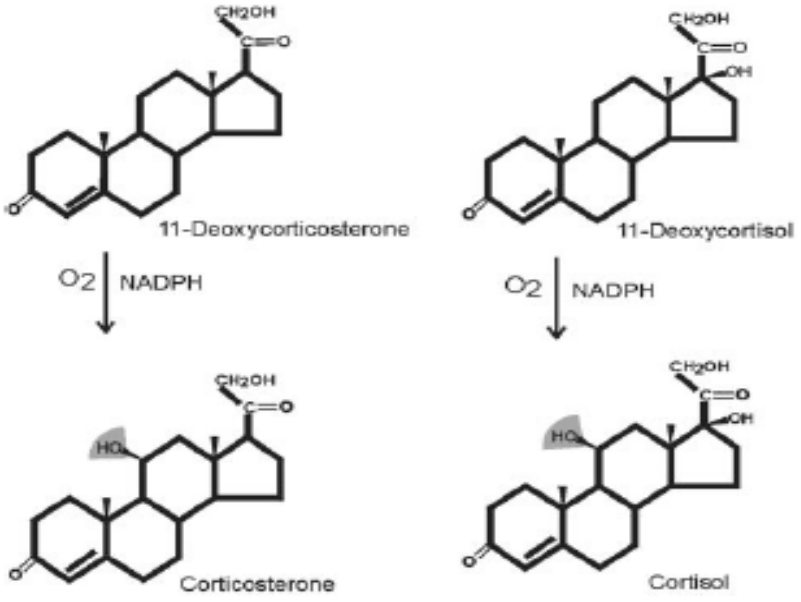


Figure 4.10. Enzymatic reactions of CYP11B1 [reproduced from Payne and Hales, 2004].

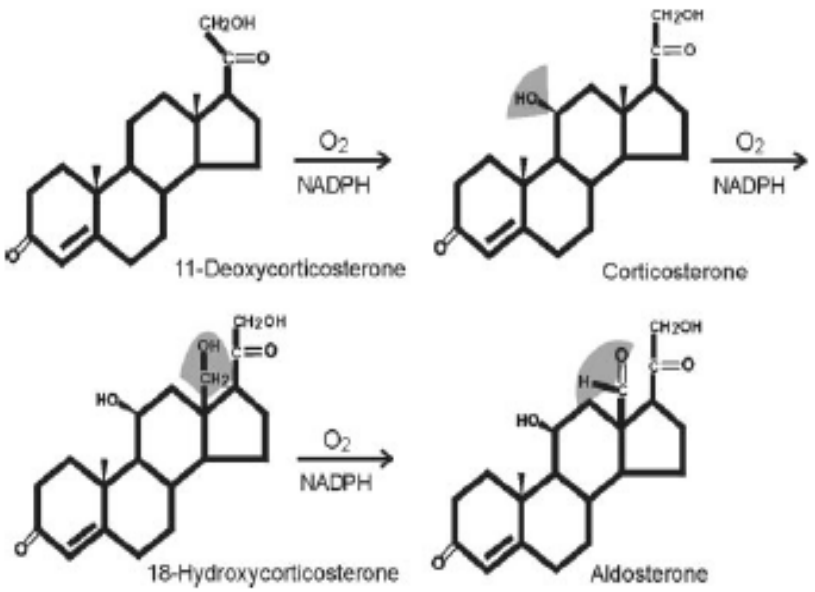


Figure 4.11. Enzymatic reactions of CYP11B2 [reproduced from Payne and Hales, 2004].

4.5 Regulation of steroidogenic cytochrome P450 enzymes

A cell's capability to synthesise specific steroids is dependent on the enzymes expressed in a particular steroidogenic cell and the stimulation thereof. A rapid rise in blood levels of adrenocortical steroids is observed in response to stress conditions that stimulate ACTH secretion [Munck *et al*., 1984]. The plasma levels of both cortisol and aldosterone in human subjects increased several fold within one hour after being injected with ACTH [Lashansky *et al*., 1991]. While cortisol secretion is mainly determined by ACTH, aldosterone secretion is affected by multiple factors secondary to ACTH stimulation. Cytokines, like interleukin-1, produced by cells of the immune system can also stimulate the HPA axis [Bahr *et al*., 1991; Lashansky *et al*., 1991]. The rapid increase in steroid secretion in response to ACTH, or other factors, is probably the result of stimulation of steroidogenesis via increased cholesterol mobilization and transport into the mitochondria [Jefcoate *et al*., 1992].

HPA axis activation is fundamental in coping with stress and disease [Munck *et al*., 1984]. During chronic stress, the continued stimulation of the adrenal cortex can increase the ability of the adrenal cortex to produce and secrete glucocorticoids by increasing the levels of key

steroidogenic enzymes. In certain disease states, blood glucocorticoid levels are elevated while adrenal androgen levels are suppressed [Nickels and Moore, 1989; Lephart *et al*., 1987]. It has been demonstrated that ACTH plays an important role in the regulation of cortisol secretion [Bahr *et al*., 1991] and that the activation of the HPA axis is essential in dealing with stress and disease [Munck *et al*., 1984].

Trophic hormones (ACTH in the adrenal zona reticularis and zona fasciculata; LH in ovarian theca, corpus lutea and testicular Leydig cells; and FSH in ovarian granulosa cells) regulate the induction of steroidogenic enzymes by binding to specific receptors on the cell surface and subsequently activating intracellular signal transduction systems. Steroid hormone receptors are proteins with specific binding sites for specific steroids. Upon ligand binding receptors undergo a conformational change which renders them activated after which they recognize and bind to specific nucleotide sequences of hormone-response elements in the promotor region of specific genes. When these ligand-receptor complexes interact with DNA, they change the transcriptional level of the associated gene by either activating or suppressing the gene that is controlled by the specific promoter.

The hormone receptors that have been characterized belong to the superfamily of G-protein linked receptors [Hanukoglu, 1992]. The interaction of hormone bound receptor with specific G-proteins activates certain membrane bound enzymes, e.g. adenylate cyclase and phospholipases, causing a brief increase in the intracellular levels of second messengers such as cAMP, cGMP, inositol triphosphate, Ca^{2+} and diacylglycerol. In the cytoplasm the hormone signal is transmitted by these small regulatory molecules that either activate protein kinases, phospholipases or interact directly with other proteins. The fundamental cellular response to hormones is generally achieved by protein kinases, which phosphorylate proteins and affect their function [Cohen, 1992; Nishizuka, 1992].

In 1992, two laboratories identified a major nuclear factor that determines cell-specific expression of P450 steroidogenic enzymes in the gonads and in the adrenal gland. Lala *et al*. (1992) named this nuclear DNA-binding protein SF-1, while Morohashi *et al*. (1992) referred to

it as Ad4BP [Lala *et al*., 1992; Morohashi *et al*., 1992]. SF-1 is part of the orphan nuclear receptor family and binds to variants of an AGGTCA sequence motif that is present in the proximal promoter of all P450 steroidogenic enzymes [Parker and Schimmer, 1995]. Even though SF-1 is vital for cell-specific gonadal and adrenal expression, there are other factors that are required for determining both maximal and cell-specific expression of these P450 enzymes. In human and mouse, adrenal-specific expression of the CYP21 genes is mediated by cell-specific elements that are located in the corresponding C4B and C4A genes [Wijesuriya *et al*., 1999; Milstone *et al*., 1992]. This distant promoter of the human CYP21 gene contains three SF-1 sites that have been identified.

Only chronic stimulation by peptide hormones, which includes ACTH acting via G protein-coupled receptors, activates adenylate cyclase thus causing a momentary increase in the level of the second messenger, cAMP, which in turn leads to the increased synthesis of steroidogenic P450 enzymes in these cells. However, the cAMP-responsive element (CRE) is not present in many of the steroidogenic P450 genes. The regulation of hormonal stimulation via cAMP leading to an increase in synthesis of these enzymes is thus not mediated via the cAMP-responsive element (CRE)/CRE binding protein (CREB) system [Todd *et al*., 1991] alone. With the exception of CYP17 whose expression appears to be entirely dependent on hormone-stimulated cAMP increases, there are additional factors involved in maintaining maximal expression of all the steroidogenic P450 enzymes [Waterman, 1994; Anakwe and Payne, 1987].

It can be concluded that although cAMP may play a role in the mechanism of action of steroidogenic P450 gene induction by trophic hormones such as ACTH, many pieces of the puzzle are still missing.

4.6 Summary

In the field of enzymology, no other enzyme has developed as dynamically as the P450 enzymes due to key functions of these enzymes in the metabolism of endogenous substrates, e.g steroid and fatty acids, as well as in the biotransformation of xenobiotics. Steroidogenic P450 enzymes

are responsible for the biosynthesis of various steroid hormones including glucocorticoids and mineralocorticoids. The most important steroidogenic tissue in the human body is the adrenal gland and, unlike the gonads, it is crucial for survival. The production of steroids is regulated by specific external stimilu, e.g ACTH, which mainly increases glucocorticoid production by acting via the cAMP-mediated protein kinase A pathway to increase P450 gene transcription [Sanderson, 2006].

During chronic stress, the negative feedback regulation of ACTH is suppressed causing a permanent elevation in the basal levels of cortisol. The detrimental effects of permanently elevated cortisol levels can be alleviated by inhibiting key steroidogenic enzymes including CYP17 and CYP21. The catalytic activity of these two enzymes thus plays an essential role in the control of adrenal steroidogenesis. The influence of rooibos extracts on the catalytic activity of CYP17 and CYP21 was thus investigated to determine whether rooibos tea could exhibit stress relieving properties by inhibiting specific pathways in adrenal steroid biosynthesis.

Chapter 5

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

5.1 Introduction

Rooibos is becoming increasingly popular partly due to its being marketed as a healthy, caffeine-free beverage with high antioxidant levels and low tannin content. Rooibos is recommended for a variety of stress-related conditions such as depression, sleep disturbances and anxiety which have been linked to the endocrine system. These symptoms are characterized by elevated glucocorticoid levels which affect behavioral responses to stress and influence various physiological functions. The biosynthesis of glucocorticoids, cortisol and corticosterone, is catalysed by the P450 enzymes in the human adrenal gland. The P450 enzymes are therefore crucial to endocrine function.

The culture of using herbal remedies derived from indigenous plants is well established in South Africa. Validating these remedies entails, amongst others, characterising biologically active compounds and using reliable scientific assays. Although the antioxidant and antimutagenic capacity of rooibos have been thoroughly studied, its influence on the endocrine system remains to be elucidated. In this study, the influence of Rooibos on two key P450 enzymes in the steroidogenic pathway, CYP17 and CYP21, was investigated to ascertain whether Rooibos is exhibiting its anti-stress properties via its influence on the adrenal steroidogenic pathway. The biological activity of Rooibos was determined by investigating the interaction of extracts with the P450 enzymes and their substrates.

The integrity of the three dimensional structure of the P450 enzymes is crucial to the activity and specificity of these enzymes. An enzyme inhibitor acts as a ligand and binds specifically to an enzyme in such a way that it may influence the ability of the enzyme to bind its substrate and

convert it to product. Inhibition studies often tell us something about the specificity of an enzyme and the physical and chemical properties of the active site. Inhibition of an enzyme by inhibitors may be either reversible or irreversible. Reversible inhibitors bind non-covalently to the enzyme and are divided into the following categories below:

Competitive reversible inhibitors are structurally related to the substrate and therefore bind to the same site as the substrate, preventing the substrate from binding. The affinity of the enzyme for the substrate decreases resulting in a higher K_m value with no change in V_{max}.

A non-competitive inhibitor binds to the unbound enzyme or the enzyme-substrate complex, at a site different from that of the substrate. Although the inhibitor does not interfere with the binding of the substrate, a dead-end complex is produced that cannot be converted to product. The V_{max} will decrease in the presence of a non-competitive inhibitor and the K_m value will remain unchanged since the enzyme forms that bind to the substrate, (unbound enzyme or enzyme-inhibitor complex) have the same affinities for the substrate at any inhibitor concentration.

Uncompetitive inhibitors only bind to the enzyme-substrate complex and not to the free enzyme, so that inhibitor binding may be at a site created by the binding of the substrate to the active site or it may bind directly to the substrate. The resulting inhibitor enzyme-substrate complex is also a dead-end complex. The V_{max} is lower in the presence of an uncompetitive inhibitor and the K_m also decreases.

Mixed type inhibitors exhibit a combination of the above effects by binding with different affinities to the free enzyme and enzyme-substrate complex, partly reducing the affinity of the enzyme for the substrate and partly reducing the maximum reaction rate.

Irreversible inhibitors bind covalently to the enzyme and inhibition involves reactions with a functional group such as hydroxyl or sulphydryl or with a metal atom in the active site or at a

distinct allosteric site. A compound that binds irreversibly with an enzyme may resemble a noncompetitive inhibitor as the V_{max} is decreased but the K_m remains unchanged.

The unique spectral properties of P450 enzymes were employed to study the influence of the unfermented and fermented rooibos aqueous and methanol extracts on the binding of PROG, the natural substrate, to adrenal microsomal P450 enzymes, CYP17 and CYP21. Using the same assay, the influence of unfermented methanol fractions on substrate binding were also investigated to determine whether the inhibitory effects observed could be attributed to compounds present in certain fractions. The degree of binding inhibition can thus be measured at various substrate and extract concentrations and calculated. The influence of extracts on the binding of substrate to the enzyme may be interpreted using the kinetic parameters applicable to the different types of inhibition discussed above so as to indicate the type of inhibtion - $\Delta Abs = B_{max}[S]/K_s + [S]$ where ΔAbs = the amplitude of the binding spectrum, B_{max} = maximum substrate binding capacity of enzyme and K_s = substrate dissociation constant.

Since binding is not necessarily indicative of catalytic inhibition, conversion of specific substrates by these enzymes was subsequently also investigated. The biological activity of the unfermented and fermented rooibos extracts were determined by investigating the effect of the extracts on the conversion of cortisol precursors. Two assays were conducted to determine the influence of rooibos extracts on the catalytic activity of CYP17 and CYP21: 1) the conversion of PROG and PREG in ovine adrenal microsomes and 2) the conversion of PROG by CYP17 and by CYP21 expressed in COS1 cells. CYP17 is at a key branch point of glucocorticoid and androgen biosynthesis in the adrenal steroidogensis pathway. Inhibition of the catalytic activity of CYP17 and CYP21 by rooibos extracts could have an influence on steroid hormone metabolism, influencing cortisol biosynthesis and thus the outcome of adrenal steroidogenesis.

5.2 Materials and methods

5.2.1 Materials

Unfermented and fermented rooibos tea was provided by Benedict Technology Holdings 75 (Pty) Ltd. Rooibos standard compounds, luteolin, vitexin, orientin, isoorientin, isovitexin and rutin were donated by Prof Wentzel Gelderblom from MRC, Western Cape. Sheep adrenals were obtained from Paarl Abattoir in Paarl, Western Cape, South Africa. The surgically removed adrenals were immediately placed on ice before use. Radioactive titrated steroids [^{3}H] PROG and [^{3}H] PREG were purchased from PerkinElmer Life Sciences (Boston, MA, USA). PROG, isocitrate and isocitrate dehydrogenase were purchased from Sigma Chemical Co. (St.Louis, MO, USA). NADH, NADPH and bovine serum albumin (BSA) were purchased from Roche Diagnostic (Germany). A Pierce BCA™ protein assay kit was purchased from Pierce (Rockford, IL, USA). Scintillation fluid was purchased from Beckman Coulter Inc. (USA).

Dulbecco's modified Eagle's medium was supplied by Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum was purchased from Highveld Biological (Lyndhurst, SA). Penicillin-streptomycin and trypsin-EDTA were purchased from Gibco-BRL (Gaithersburg, MD USA). COS-1 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). TransIT®-LTI Transfection reagent was purchased from Wizard® *Plus* SV Midipreps and DNA Purification kits were purchased from Promega Biotech (Madison, WI, USA). All other chemicals were of the highest quality and purchased from reputable scientific supply houses.

All spectra were recorded using a Varian Cary 100 double-beam UV-Vis spectrophotometer. Chromatography was performed on a Waters (Milford, MA, USA) high performance liquid chromatograph coupled to a P4000 (Thermo Separation Products, San Jose, USA) automatic injector and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL) and a Waters absorbance detector (254nm).

5.2.2 Preparation of unfermented and fermented Rooibos tea extracts

An aqueous extract of unfermented rooibos was prepared by adding 100 ml deionised water (100°C) to 5 g of plant material and boiling the mixture on a hot plate for 30 minutes. The extract was filtered using a Whatman No.4 filter paper and an 0.45 µm pore size filter before lyophilizing. An aqueous extract of fermented rooibos was prepared in the same way. After the unfermented and fermented extracts were lyophilized, yielding 0.687 g and 0.680 g plant material respectively, the dried material was resuspended in 8 ml deionized water and stored at -20°C.

A methanol extract was prepared by placing 30 g unfermented rooibos plant material in a glass soxhlet extractor fitted with a double wall condenser. The extractor was fitted to a round bottom flask containing 300 ml methanol and the plant material was extracted for ≈8 hours, until the effluent from the soxhlet extractor was clear. The methanol extract was dried under vacuum on a rotary evaporator, yielding 3.15 g dried plant material. The dried extract was resuspended in 35 ml deionized water and centrifuged at 6000 g for 5 min. Undissolved plant extract was removed using an 0.45 µm pore size filter and stored at -20°C in 5 ml aliquots. A methanol extract of fermented rooibos was prepared in the same way yielding 3.18 g of dried plant material. The final concentration of unfermented aqueous and methanol extract were 92 mg/ml and 90 mg/ml respectively. The final concentration of the fermented aqueous and methanol extracts were both 91 mg/ml.

5.2.3 LC-MS of unfermented and fermented rooibos methanol and aqueous extracts

LC-MS was performed with a Waters API Q-TOF Ultima mass spectrometer. A capillary voltage of 3.5kV was applied with a source temperature of 100 ºC. The cone voltage was set at 35V. Data acquisition was in the positive mode through m/z = 100 – 1999 scan range. A 270 µl injection of the unfermented and fermented rooibos methanol samples was separated on a Waters Alliance UPLC fitted with a Phenomenex Gemini C18 column (1.7µm, 2.1 mm x 50mm).

Solvent A, deionised water containing 1 % formic acid, and Solvent B, 100% acetonitrile, was used to obtain the desired separation, with the gradients shown in Table 5.1. A flow rate of 1ml/min was applied.

Table 5.1: Gradient table for HPLC separation of unfermented and fermented rooibos methanol extracts.

Time	Solvent A	Solvent B
0.00	98	2
30.00	0	100
35.00	0	100
35.01	98	2
45.00	98	2

5.2.4 Fractionation of an unfermented rooibos methanol extract

An HPLC method was developed to fractionate the unfermented rooibos methanol extract using a Waters (Milford, MA, USA) high performance liquid chromatograph coupled to a Waters 991 Photodiode Array detector. A Novapak® C18 reverse phase column (1.7µm, 2.1 mm x 50mm) was used and the elution system consisted of solvent A, deionised water, and solvent B, methanol. A linear gradient was run from 100 % A to 100 % B in 30 minutes at a flow rate of 2.5 ml/min. The eluate was monitored at 420 nm and five distinct peaks were identified and subsequently collected. The fractions were lyophilized and resuspended in deionized water to a final concentration of 2.5 mg/ml. The five fractions were subsequently subjected to LC-MS and their profiles compared to that of standard flavonoid compounds under the same chromatographic conditions.

5.2.5 LC-MS of unfermented rooibos methanol fractions

LC-MS of the unfermented rooibos methanol HPLC fractions was performed on a Waters API Q-TOF Ultima mass spectrometer. A capillary voltage of 3.5kV was applied with a source temperature of 100 ºC. The cone voltage was set at 35V. Data acquisition was in the positive mode through m/z = 250-1200 scan range. The fractions were separated on a Waters Alliance UPLC fitted with a Phenomenex Gemini C18 column (1.7µm, 2.1 mm x 50mm). Flavonoid

compounds, luteolin, vitexin, orientin, isoorientin, isovitexin and rutin, were analysed under the same conditions. Solvent A, deionised water containing 1 % formic acid, and Solvent B, 100% acetonitrile, was used to obtain the desired separation, with the gradients shown in Table 5.2. A flow rate of 0.2 ml/min was applied.

Table 5.2: Gradient table for HPLC separation of unfermented rooibos methanol fractions

Time	Solvent A	Solvent B
0.00	85.0	15.0
15.00	82.0	18.0
20.00	60.0	40.0
24.00	10.0	90.0
25.00	0.0	100.0
25.10	85.0	15.0

5.2.6 Preparation of adrenal microsomes

Microsomal fractions were prepared as previously described using adrenals obtained from newly slaughtered sheep [Yang and Cederbaum, 1994]. All procedures were performed at 4°C. Decapsulated adrenals were washed in 1.15 % KCl after which cortex tissue (25 g) was homogenized in 75 ml 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 0.25 M sucrose, first in a Hamilton Beech blender and subsequently with a Potter-Elvejhem glass homogenizer. The homogenate was centrifuged at 1000 x g for 20 min. The pellet was discarded and the supernatant was centrifuged at 12000 x g for 15 min. The resulting pellet was discarded and PEG 8000 (50 % w/v) was slowly added to the supernatant containing the microsomal fraction to a final concentration of 8.5 %. The mixture was stirred for 10 min and centrifuged at 13000 x g for 20 min. The supernatant was discarded and the microsomal pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl and 1.0 mM EDTA. PEG (50 % (w/v)) was added to the suspension to a final concentration of 8.5 % and the mixture was stirred for 10 min and centrifuged at 13000 x g for 20 min. This step was repeated twice until the supernatant was clear. The final microsomal pellet was resuspended in 80 ml 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA and stored at -80°C in 5 ml aliquots.

5.2.7 Determination of cytochrome P450 concentration

The cytochrome P450 content of the adrenal microsomes was determined by the carbon monoxide method described by Omura and Sato [Omura and Sato, 1964]. The microsomal suspension was diluted, 1:3, in 0.1 M phosphate buffer (pH 7.4) containing 10 % ethylene glycol. This volume was subsequently saturated with carbon monoxide and divided into two optically matched quartz cuvettes. A baseline was recorded between 400 and 500 nm. Sodium dithionate (1-2 μg) was added to the sample cuvette and the spectrum recorded. Spectra were monitored for ~10 min until the spectrum was completely developed.

The cytochrome P450 concentration (c) was determined by using the molar extinction coefficient (ε) of 91 $cm^{-1}mM^{-1}$, as reported by Omura and Sato, from the equation $\Delta A = \varepsilon C l$ [Omura and Sato, 1964].

The protein concentration of the microsomal preparation was determined using the Pierce BCA Protein Assay Kit (Rockford, IL, USA).

5.2.8 Bioactivity assays

A. Spectral binding assay

The interaction of rooibos extracts with P450 enzymes was investigated by assaying the binding of PROG to CYP17 and CYP21 in the presence of methanol and aqueous extracts of fermented and unfermented rooibos. The binding of PROG and 17OH-PROG was subsequently assayed in the presence of varying concentrations of unfermented aqueous fractions.

Substrate-induced difference spectra

Reactions were carried out at room temperature in a final reaction volume of 1 ml. The cytochrome P450 concentration of the microsomal suspension used in the assay was 0.8 μM. The microsomal preparation was pipetted into two optically matched quartz cuvettes and a

baseline recorded between 370 and 450 nm. The steroid substrate, PROG or 17OH-PROG, dissolved in ethanol was added to the sample cuvette with an equal volume of ethanol added to the reference cuvette. The cuvettes were inverted gently to mix the contents and substrate-induced difference spectra were recorded at final substrate concentrations of 2, 4, 8, 16 and 32 μM.

Influence of rooibos extracts on substrate- induced difference spectra

Substrate binding assays were conducted in the presence of aqueous and methanol extracts of unfermented and fermented rooibos to determine the influence of rooibos on the binding of endogenous steroids to CYP 17 and CYP21. Substrate-induced difference spectra of CYP17 and CYP21 were obtained with PROG as a substrate. To assay the inhibition, 8 μl extract was added per 1 ml microsomal suspension, mixed and divided into two optically matched cuvettes afterwhich the baseline was recorded. The final concentration of the methanol and aqueous extracts of unfermented rooibos was 0.72 mg/ml and 0.74 mg/ml respectively and 0.73 mg/ml for both the fermented methanol and aqueous extracts. The steroid substrate, PROG (16 μM), was added to the sample cuvette with an equal volume of ethanol added to the reference cuvette and substrate-induced difference spectra were recorded as described above.

Once the influence of aqueous and methanol extracts of unfermented and fermented rooibos on the binding of PROG was established the inhibitory effect of different concentrations of the aqueous unfermented rooibos extract on the steroid-induced difference spectra was investigated. The extract, 2 μl – 8 μl was added to microsomal preparations to a final concentration of 0.18 mg/ml – 0.74 mg/ml. The steroid substrates, PROG and 17OH-PROG (final concentrations ranging from 2 to 32 μM) were added to sample cuvette and substrate-induced difference spectra were recorded between 370 and 460 nm as described above.

The inhibitory effect of unfermented methanol rooibos HPLC fractions on the binding of PROG to the microsomal P450 enzymes was determined. The fractions were assayed using a final

concentration of 0.08 mg/ml. Substrate-induced difference spectrum were obtained using PROG (16 μM) as a substrate as described above.

Inhibition was indicated by a reduction in the amplitude of the peaks – a decrease in the absorbance maximum at 383 nm and an increase in the absorbance minimum at 416 nm. The inhibitory effect may be calculated as follows:

$$\% \text{ Inhibition} = 100 - ((t/c) \times 100)$$

where t = absorbance at 390 nm minus absorbance at 420 nm in the presence of inhibitory component and c = absorbance at 390 nm minus absorbance at 420 nm in the absence of inhibitory component.

B. Steroid conversion assays

The conversion of PREG and PROG by microsomal enzymes CYP17 and CYP21 was initially examined in the presence of aqueous and methanol extracts of unfermented and fermented rooibos. Subsequent investigations were carried out in COS1 cells expressing either CYP17 or CYP21 to ascertain the inhibitory effect of unfermented and fermented rooibos methanol extracts on the individual enzymes. Following the fractionation of the methanol extract of unfermented rooibos, PROG conversion by CYP17 and CYP21 was assayed in COS1 cells in the presence of the collected fractions. Flavonoid compounds indentified in these fractions were subsequently also assayed in COS1 cells expressing CYP21.

Steroid conversion assays in adrenal microsomes

Steroid substrate conversion assays were conducted to determine if the metabolism of PROG and PREG by microsomal P450 enzymes was influenced in the presence of unfermented and fermented rooibos aqueous and methanol extracts. The metabolism of PREG and PROG was examined in ovine adrenal microsomes as previously described [Swart *et al*., 2003]. The assay was carried out in a shaking water bath at 37°C in a final reaction volume of 1 ml. Radiolabeled tritiated steroid solution (100 000 cpm/50 μl) and steroid solution (10 μM) was pipetted onto filter paper, placed in a microcentrifuge tube and dried under nitrogen. Once the organic solvents

had evaporated, isocitrate (2 mg/ml), $MgCl_2$ (10 mM), 50 mM Tris buffer (pH 7.4), containing 50 mM NaCl and 1 % BSA, and the microsomal preparation (0,35 µM P450) were added to the dried steroids. The resulting mixture was incubated for 5 min at 37°C before the reaction was initiated by the addition of NADPH (1 mM) and isocitrate dehydrogenase (0.8 U/ml). Trilostane (20 µM), a 3βHSD inhibitor, was added to the reaction mixture when PREG was used as a substrate.

The effect of the extracts on PROG and PREG metabolism was determined by the addition of 25 µl of each extract (25 µl buffer or solvent in the control assay) to the microsomal reaction mixture prior to the initiation of the reaction. The final concentration of extracts was 2.25 mg/ml and 2.28 mg/ml respectively for the unfermented methanol and aqueous extracts respectively and 2.3 mg/ml for both the fermented methanol and aqueous extracts. Aliquots, 50 µl, were removed before the reaction was initiated as well as after 15 min and pipetted into glass screw cap tubes containing a cold (4°C) mixture of 5 ml dichloromethane (10 volumes) and 450 µl deionized water. The steroid metabolites were extracted by vortexing the mixture for 2 min after which the water phase was aspirated and the dichloromethane subsequently evaporated under nitrogen. The steroid products were redissolved in 120 µl methanol prior to HPLC analysis.

Steroid conversion assays in COS1 cells

Since microsomal preparations contain both CYP17 and CYP21, the effect of rooibos on each enzyme was determined using COS1 cells, a non-steroidogenic mammalian cell line and expressing the recombinant enzymes individually in the presence of rooibos.

5.2.9 Maintenance of COS1 cells

COS1 cells were grown at 37°C with 5 % CO_2 and 90 % humidity. The addition and removal of media were conducted in sterile conditions in a laminar flow hood. A freezing vial containing 1 ml COS1 cells was thawed at room temperature and resuspended in 10 ml culture media (DMEM, high glucose containing 0.15 % $NaHCO_3$, 10 % fetal calf serum and 1 % penicillin

streptomycin). The cell suspension was plated out in a 100 mm tissue culture dish and incubated, replacing the culture media every day. Confluent cells were split in a ratio of 1:3. Briefly, the medium was removed from the culture dish and the dish was washed with 1 ml warm trypsin media. The cells were incubated for three minutes at room temperature in 1 ml trypsin media before the cells were collected and transferred to a conical tube containing 30 ml of culture media. The cells were resuspended and plated out into three dishes and cultured as described above.

Freezing cells

Freezer stocks were prepared by collecting and transferring the cells to 50 ml conical tubes as described above. The cell suspension was centrifuged for 5 min at 1200 rpm and the supernatant was removed. The cell pellet was resuspended in culture media containing 10 % DMSO (3 ml per 100 mm dish). The cell suspension, 1 ml, was pippetted into cryovials, and frozen for 48 hours at -80°C before being transferred to liquid nitrogen where it was stored for future use.

Transfection of COS1 cells

The cells were grown to confluency and split one day prior to transfection. The cells were split into 12 well plates (3.8 cm^2) with each well containing approximately $1x10^5$ cells, incubated for 24 h after which the culture media was removed replaced with 1 ml fresh media.

Serum-free culture media (50 µl per well) was pipetted into a sterile microcentrifuge tube and incubated with the transfection reagent (1.5 µl per well) for 10 min at room temperature. Plasmid DNA (0.5 µg per well) was added to the mixture and incubated for a further 20 min at room temperature after which 50µl was added to each well.

Cells were transfected with the following vector constructs: baboon CYP17/pTarget and baboon CYP21/pTarget expressing recombinant CYP17 and CYP21 respectively; angora

CYP17/pVector expressing angora CYP17 (positive control); and pCI-neo,containing no DNA insert (negative control). Cells were incubated for 72 h before the addition of substrate.

Metabolic assay

Substrate conversion assays were initiated 72 h after transfection by replacing the culture media with media containing steroid substrate, 1 μM, together with the tritiated steroid ($2x10^6$ cpm/ml) in the absence and in the presence of extracts and fractions. The cells were incubated for 24 h where after 50 μl of culture media was removed and the steroid metabolites extracted as previously described. The steroid products were resuspended in 120 μl methanol for HPLC analysis.

The unfermented and fermented rooibos methanol extracts comprised 50 μl of the 1 ml substrate media. The final concentrations of the unfermented and fermented rooibos methanol extracts in the assay were 4.5 mg/ml and 4.6 mg/ml respectively. The unfermented methanol fractions of rooibos comprised 200 μl of the substrate media with a final concentration of 0.5 mg/ml.

Flavonoid compounds, rutin, vitexin and orientin, comprised 3 μl, 48μl and 30 μl respectively of the substrate media to yield a final concentration of 3 μM for each compound. A final concentration of 10 μM for each compound, rutin, vitexin and orientin was added in 10 μl, 130 μl and 99 μl of the substrate media respectively. No tritiated substrate was added to the culture media as the steroid substrate, PROG, and its metabolite, DOC, was analysed using LCMS.

Cell viability assay

The effect of the extracts on the viability of the COS1 cells was determined by assaying the metabolism of testosterone in the presence and absence of rooibos extracts. Although they are non-steroidogenic, COS1 cells have the ability to convert testosterone to 4-androstenedione due to the expression of 17-dehydrogenase activity. Substrate media containing testosterone (1 μM) as steroid substrate was added to confluent COS1 cells, with and without extracts. The

unfermented and fermented methanol extracts were added to the medium at final concentrations of 4.5 mg/ml and 4.6 mg/ml respectively, the same concentration used in the metabolic assays. Flavonoid compounds, rutin, vitexin and orientin, dissolved in ethylene glycol, were also added to the medium at a final concentration of 10μM for each compound. After an incubation period of 24 h, 50 μl culture media was removed, extracted and subjected to LCMS analysis.

5.2.10 HPLC of steroid metabolites

PREG metabolites were separated on a Novapak® C18 column (1.7μm, 2.1 mm x 50mm) at a flow rate of 1 ml/min. The mobile phase used to separate the steroids consisted of solvent A, 75:25 methanol/water, and solvent B, 100 % metanol. The steroid metabolites were eluted from the column for 15 min with solvent A, followed by a linear gradient from 100 % A to 100 % B in 5 min and an isocratic elution with solvent B for 3 min.

PROG metabolites were separated using the same column and flow rate described above. The mobile phase used to separate the steroids consisted of solvent A, 65:35 methanol/water, and solvent B, 100% methanol. The steroid metabolites were eluted from the column for 10 min with solvent A, followed by a linear gradient from 100% A to 100% B in 5 min and an isocratic elution with solvent B for 2 min (17).

Areas under the individual peaks of tritiated steroid metabolites detected with the radioactive flow detector were integrated and expressed as a percentage of the total radioactivity. The inhibition of substrate conversion was calculated as follows:

$$\% \text{ inhibition} = 100 - ((t/c) \times 100),$$

where t = % conversion or formation of steroid in the presence of the inhibitory component and c = % conversion or formation of steroid in the absence of inhibitory component.

5.2.11 LCMS separation of steroid metabolites

LC-MS was performed with a Waters API Q-TOF Ultima mass spectrometer. A capillary voltage of 3.5kV was applied with a source temperature of 100 ºC. The cone voltage was set at 35V. Data acquisition was in the positive mode through m/z = 315-331 scan range.
PROG and its steroid metabolite, DOC, were separated with the gradient illustrated in Table 5.3. Solvent A, deionised water containing 1% formic acid and solvent B, 100% methanol was used to obtain the desired separation with a flow rate of 0.3 ml/min.

Table 5.3: Gradient table for HPLC separation of PROG and DOC

Time	Solvent A	Solvent B
0.00	56.0	44.0
6.00	56.0	44.0
6.01	20.0	80.0
9.50	0.0	100.0
10.00	56.0	44.0
12.00	56.0	44.0

5.2.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 4.00 for Windows, GraphPad Software, San Diego, California, USA. The results are expressed as the mean with error bars representing the standard errors of the means (SEM).

5. 3 Results

The biological activity of rooibos extracts was examined by firstly investigating whether the extracts influence the binding of steroid substrates to cytochrome P450 enzymes. Ferric cytochrome P450 interacts with a number of its substrates with a concomitant shift in the equilibrium between the high- and low-spin configurations. The binding of the steroids results in the conversion of the ferric iron from a low spin state to a high spin state. Steroids binding to microsomal P450 enzymes typically induce difference spectra with PREG producing type II spectrum with an absorption maximum at 420 nm and an absorption minimum at 390 nm and

PROG producing type I spectrum with an absorption maximum at 390 nm and an absorption minimum at 420 nm. Compounds interfering with or prohibiting the binding of substrate to the P450 enzyme will reduce the amplitude of the spectrum by decreasing the absorbance maximum and increasing the absorbance minimum. If an inhibitor binds directly to the ferric iron by replacing the water molecule as the ligand at the sixth position, a type II spectrum will be induced in the absence of substrate.

The cytochrome P450 content of the microsomes isolated from ovine adrenal glands was determined by the carbon monoxide-induced difference spectra [Omura and Sato, 1964]. The P450 concentration was calculated using the millimolar extinction coefficient as described by Omura and Sato [Omura and Sato 1964]. The sodium dithionite reduced carbon monoxide spectrum of ovine adrenal microsomal cytochrome P450 is shown in figure 5.1. The reduction of cytochrome P450 is a slow reaction, even with sodium dithionate being a strong reductant, and it is thus necessary to allow the absorbance at 450 nm to completely develop.

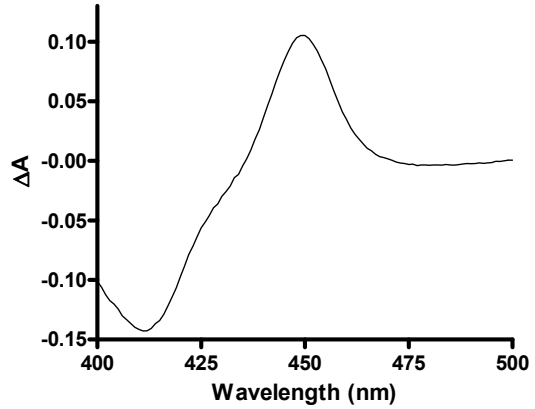
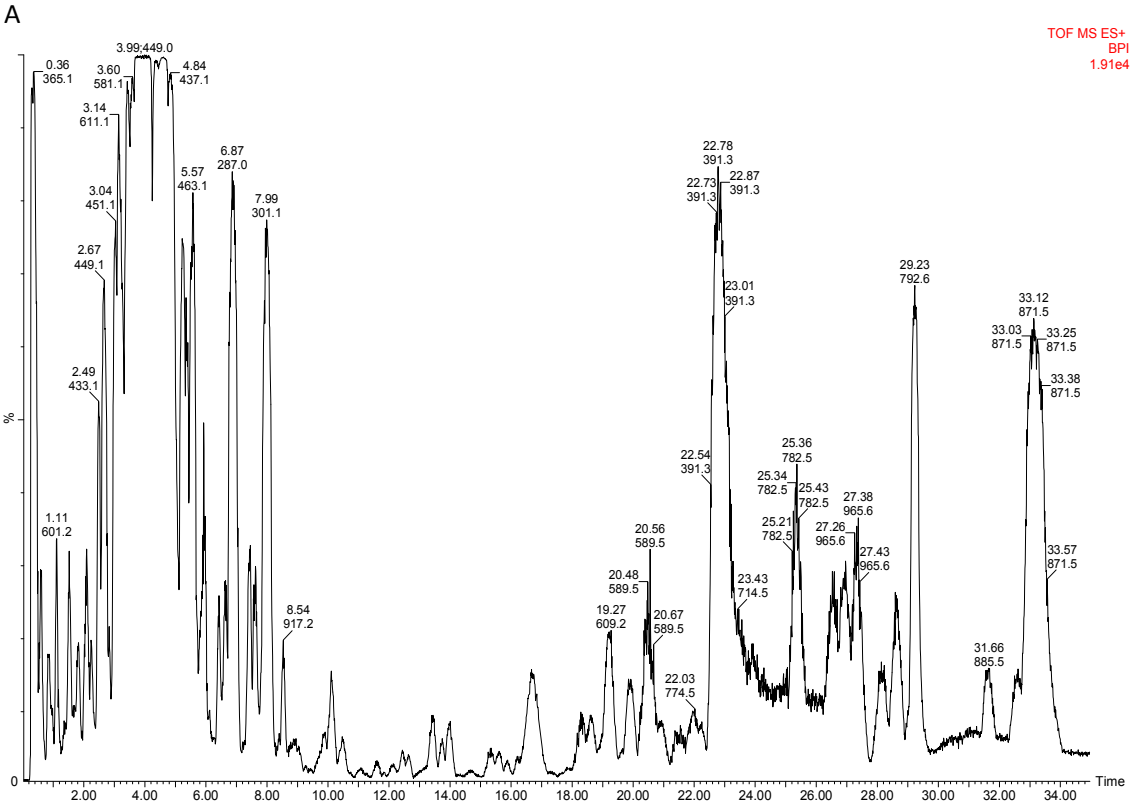


Figure 5.1. Sodium dithionate reduced carbon monoxide difference spectra of the ovine adrenal microsomal preparation. [P450] = 1.15μM (0.155 nmol/ml protein).

The concentration of ovine adrenal microsomal P450 was 1.15 μM (0.155nmol/ml protein). The microsomal preparation contained the P450 enzymes CYP17 and CYP21.

5.3.1 LCMS of fermented and unfermented rooibos methanol extracts

LC-MS analyses of fermented and unfermented rooibos methanol and aqueous extracts revealed that the extracts consist of a complex mixture of compounds (figure 5.2). Although their elution profiles are very similar, the unfermented rooibos extract yielded peaks that were absent in the fermented methanol extract. These hydrophobic compounds eluting later at 24 – 32 min have high molecular masses. Three of the most prominent peaks have molecular masses of 728, 885 and 965. No peak identification could be carried out at this stage since no standards were available at the time of the investigation. Initial studies were therefore carried out with the four extracts to determine the influence of these extracts on the P450 enzymes.



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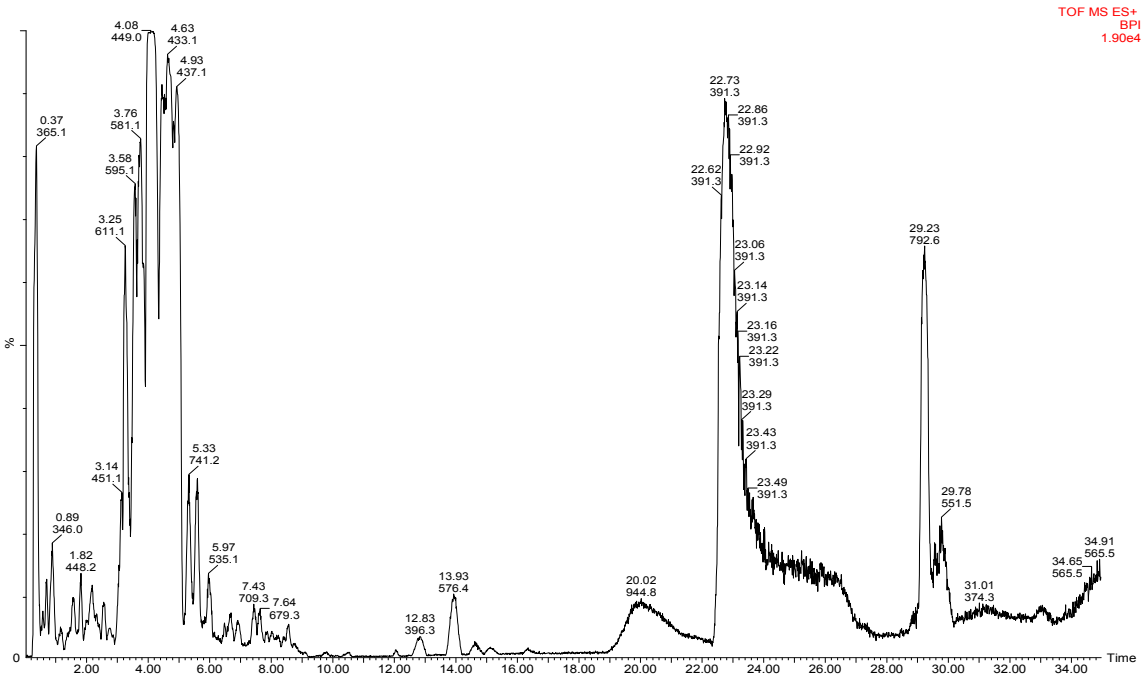
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365.1
4.08
449.0
4.63
433.1
4.93
437.1
3.76
581.1
3.58
595.1
3.25
611.1
3.14
451.1
0.89
346.0
1.82
448.2
5.33
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5.97
535.1
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709.3
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679.3
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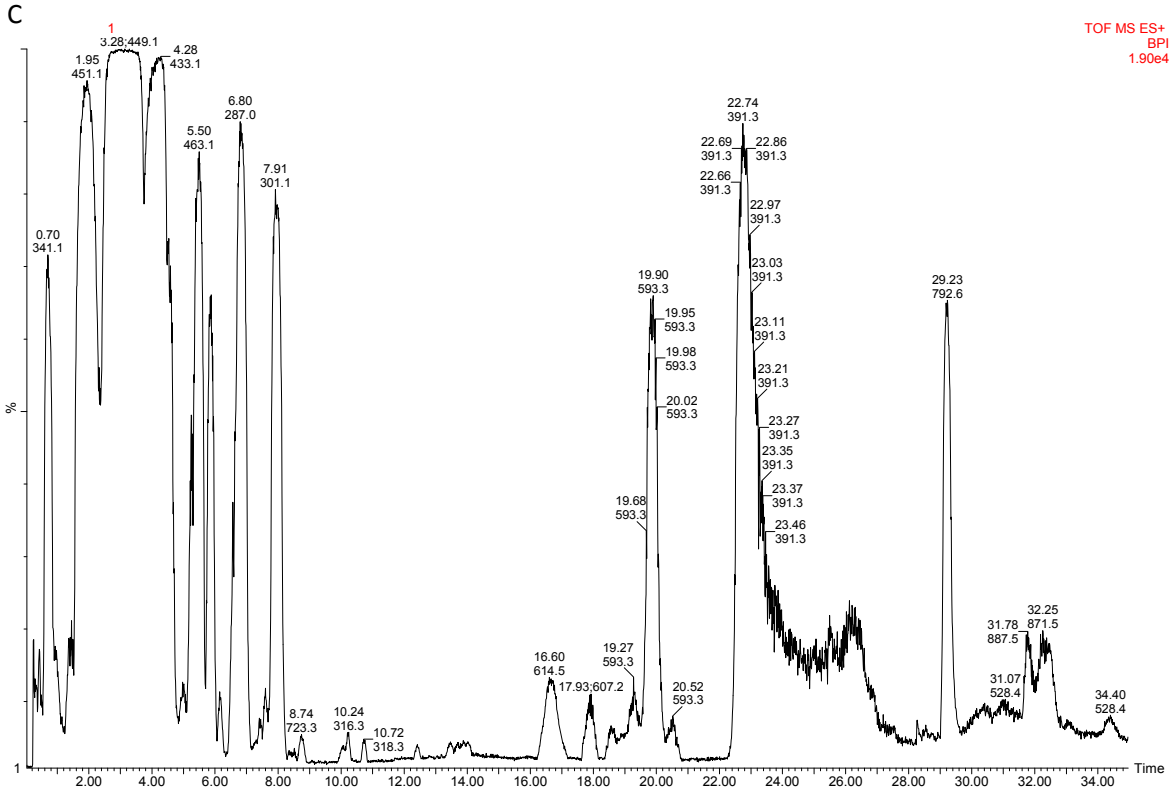
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32.00
34.00
3.28;449.1
1.95
451.1
4.28
433.1
6.80
287.0
5.50
463.1
7.91
301.1
0.70
341.1
8.74
723.3
10.24
316.3
10.72
318.3
16.60
614.5
17.93;607.2
19.27
593.3
19.68
593.3
19.90
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19.95
593.3
19.98
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391.3
22.86
391.3
22.66
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391.3
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792.6
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32.25
871.5
31.07
528.4
34.40
528.4

D

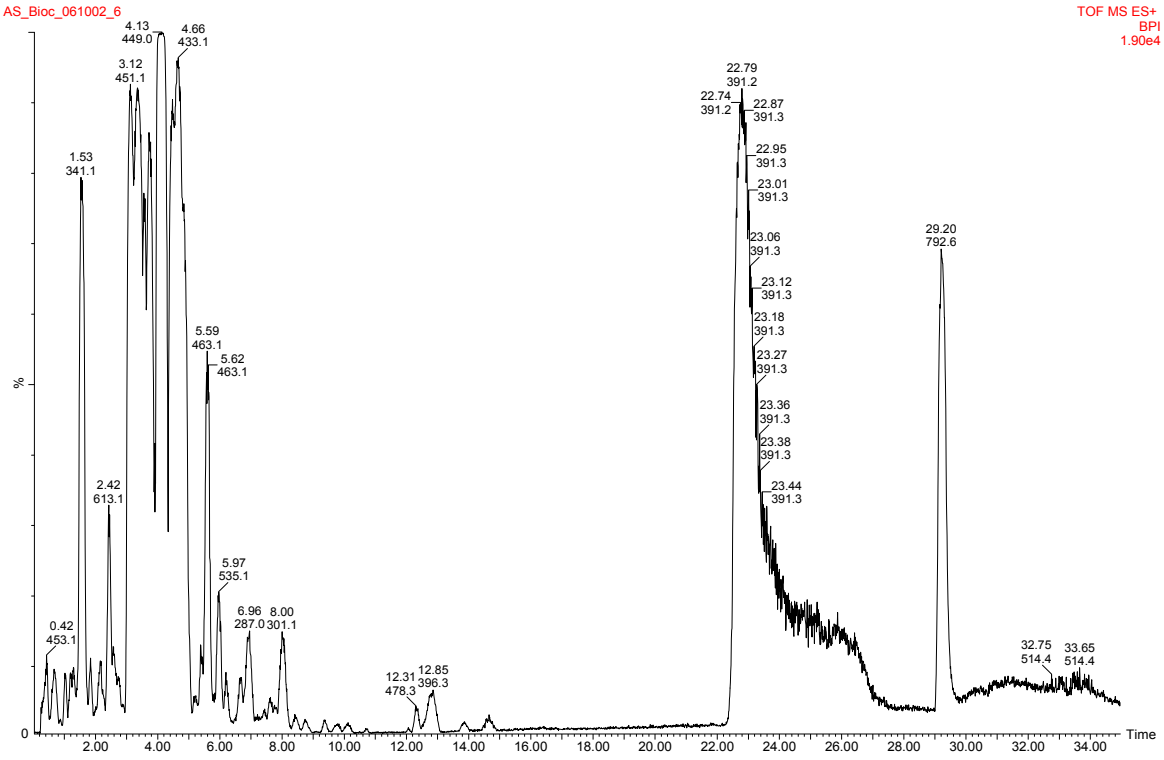


Figure 5.2. LC-MS total ion chromatogram (positive mode) analysis of unfermented rooibos A) methanol extract and B) aqueous extract and fermented rooibos C) methanol extract and D) aqueous extract.

5.3.2 Spectral assays

In previous studies in determining the influence of rooibos on P450 enzymes, carried out in our laboratory, it was shown that rooibos extracts do not inhibit the binding of PREG to the microsomal enzymes. In addition, it was not possible to assay binding of extracts to the P450 enzymes in the absence of substrate due to interference of plant pigments in the 360 – 500nm absorbance range.

PROG and 17OH-PROG bind to the microsomal P450 enzymes inducing typical type I spectra with absorption maxima at 390 nm and absorption minima at 420 nm. Inhibition was indicated by a reduction in the amplitude of the peaks, a decrease in the absorbance maximum at 390 nm and an increase in the absorbance minimum at 420 nm (figure 5.3).

In this study four extracts, methanol and aqueous extracts of fermented and unfermented rooibos, were prepared and the inhibition of PROG binding to microsomal P450 enzymes subsequently assayed. The extracts inhibited the binding of PROG with the unfermented extracts exhibiting a greater inhibitory effect that the fermented extracts (figure 5.4). The inhibition of PROG binding by the unfermented rooibos methanol and aqueous extracts was greater than 35 % ($P<0.001$).

The lowest inhibition was obtained with the fermented rooibos aqueous extract which inhibited the binding of PROG by 13 % ($P<0.05$) with the fermented rooibos methanol extract inhibiting PROG binding by 27 % ($P<0.01$). The inhibition of PROG binding by the unfermented rooibos aqueous extract was significantly higher ($P<0.001$) than the inhibition of fermented rooibos methanol and aqueous extracts. The final concentration of the unfermented rooibos aqueous and methanol extracts were 0.74 mg/ml and 0.72 mg/ml respectively. The final concentration of the fermented rooibos methanol and aqueous extracts were both 0.73 mg/ml.

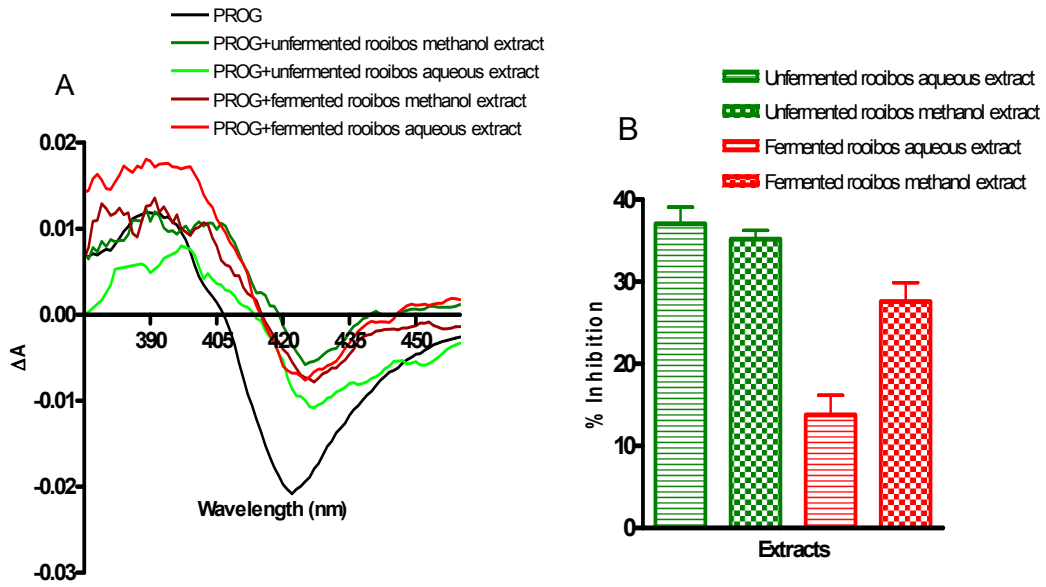


Figure 5.3. A) Inhibition of PROG-induced type I difference spectra in ovine adrenal microsomes ([P450] = 0.8 µM) and B) percentage inhibition of PROG binding in the presence of rooibos extracts. [PROG] = 16 µM; [unfermented rooibos aqueous extract] = 0.74 mg/ml; [unfermented rooibos methanol extract] = 0.72 mg/ml; [fermented aqueous extract] = 0.73 mg/ml, and [fermented rooibos methanol extract] = 0.73 mg/ml. are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

The inhibition of aqueous extracts of the unfermented rooibos on the binding of PROG and 17OH-PROG to adrenal microsomal P450 enzymes was investigated using varying concentrations of substrate (2 – 32 µM) and extracts (final concentrations, 0.184 – 0.736 mg/ml). The inhibition of PROG (2µM) and 17OH-PROG (2µM) binding to microsomal P450 enzymes in the presence of 2µl of the aqueous extract of unfermented rooibos (final concentration of 0.736 mg/ml) was 29.4 % and 10 %, respectively (figure 5.4).

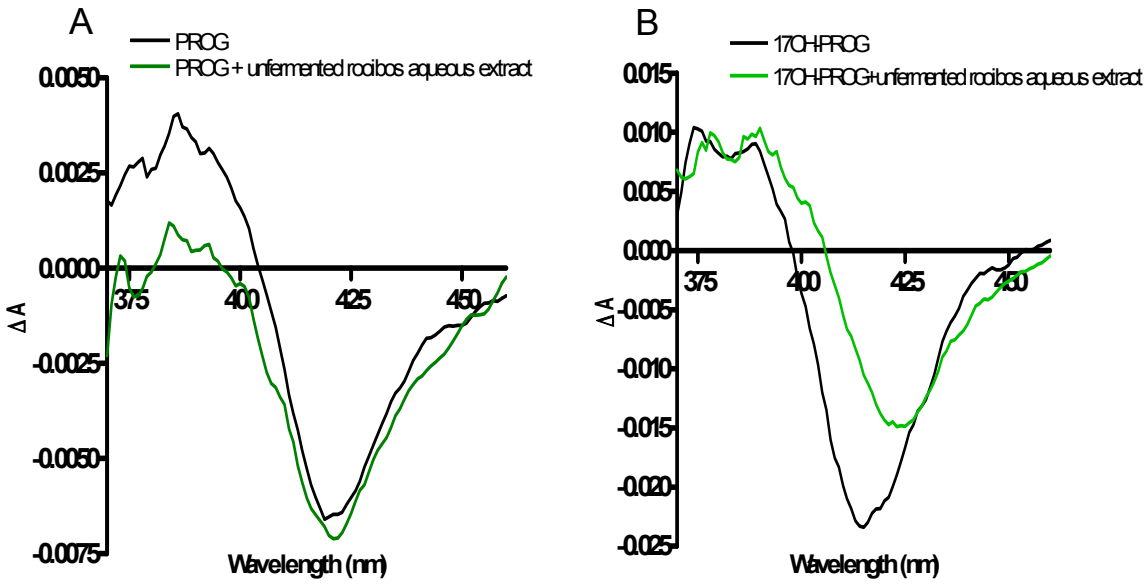


Figure 5.4. Inhibition of steroid-induced type I difference spectra in ovine adrenal microsomes. [P450] = 0.8 µM; A) [PROG] = 2 µM; [unfermented rooibos aqueous extract] = 0.184 mg/ml and B) [17OH-PROG] = 2 µM;[unfermented aqueous extract] = 0.184 mg/ml.

The data obtained from the assays determining the degree of inhibition of PROG and 17OH-PROG at various substrate and extract concentrations generated hyperbolic saturation binding curves (figure 5.5). It can be seen from the data that the maximum substrate binding capacity (B_{max}) of the microsomal enzymes differs markedly. This may be due to the fact that the microsomal preparation contains both CYP17 and CYP 21 enzymes and while PROG and 17OH-PROG are both natural substrates for CYP21, CYP17 catalyses only the conversion of PROG showing negligible catalytic activity towards 17OH-PROG.

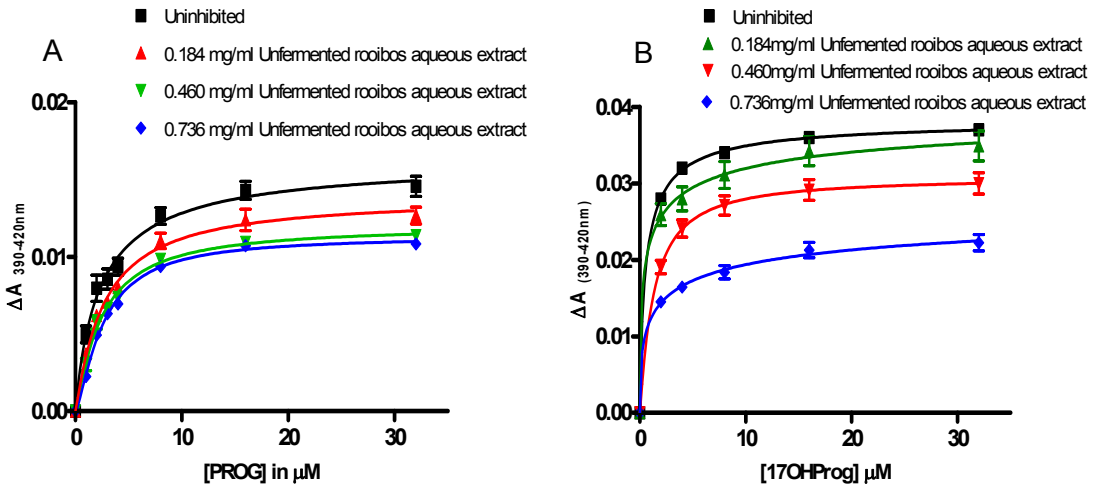


Figure 5.5. Hyperbolic saturation binding curves of die inhibitory effect of unfermented rooibos aqueous extract on A) PROG and B) 17OH-PROG binding to microsomal P450 enzymes. [P450] = 0.8 µM; [Prog] and [17OH-PROG] = 2 – 32 µM; [unfermented rooibos aqueous extracts] = 0.184 – 0.736 mg/ml. Results are presented as the mean, error bars represent SEM and n=3.

Double reciprocal plots, a convenient linearization of Michaelis-Menten data, were plotted to visualize the inhibition data. It appears that in the presence of unfermented rooibos aqueous extracts, the binding of PROG to the P450 enzymes is being competitively inhibited as only the K_i (binding inhibition constant) changes (figure 5.6). It is possible for compounds in the extracts to bind to the enzymes or to the enzyme-substrate complex and thus mixed inhibition of binding was also analysed. The secondary plots are shown in figure 5.7. Analysis of the data revealed K'_i (apparent inhibition constant for the binding of E + I) and K_i (inhibition constant for the binding of ES + I), to be 1.3 mg/ml (figure 5.7 A and B). The binding of PROG to the microsomal enzymes appears to be competitively inhibited in the presence of the concentrations assayed for unfermented rooibos aqueous extracts.

The double reciprocal plot of 17OH-PROG binding shows that 17OH-PROG is being competitively inhibited at low concentrations of extract with uncompetitive or mixed inhibition occurring at higher concentrations (figure 5.8). Analysis of the data (figure 5.9 A and B) revealed that the K_i and the K'_i values, 0.08 and 1.9 mg/ml do indicate mixed inhibition of the binding of 17OH-PROG to the microsomal enzymes by unfermented rooibos aqueous extracts.

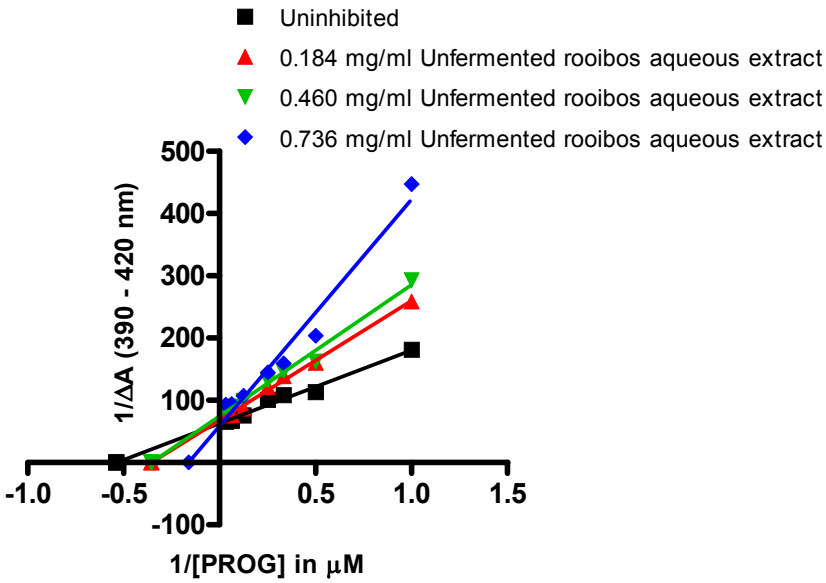


Figure 5.6. Double reciprocal plot of the inhibitory effect of unfermented rooibos aqueous extract on PROG binding. [P450] = 0.8 µM; [PROG] = 2 – 32 µM; unfermented rooibos aqueous extract = [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

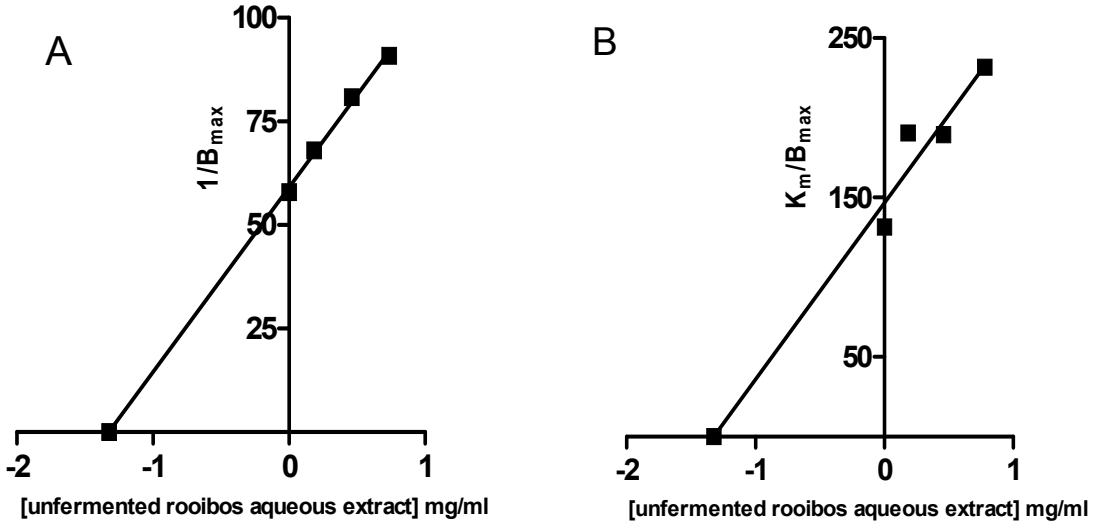


Figure 5.7. Secondary plots for inhibition of PROG binding to microsomal P450 enzymes in the presence of unfermented rooibos aqueous extract A) plot of $1/B^{app}_{max}$ against extract concentration and B) plot of K_m/B^{app}_{max}. [P450] = 0.8 µM; [17OH-PROG] = 2 – 32 µM; unfermented rooibos aqueous extract [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

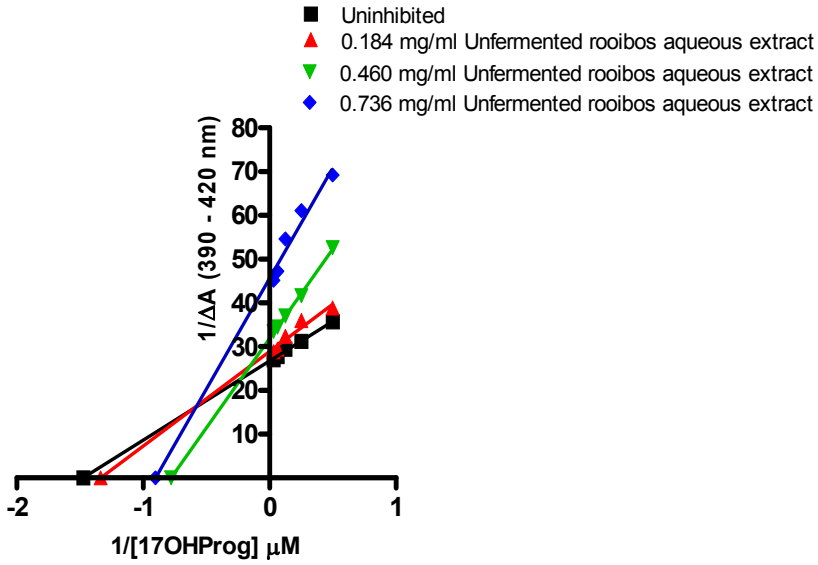


Figure 5.8. Double reciprocal plot of the inhibitory effect of unfermented rooibos aqueous extract on 17OH-PROG binding. [P450] = 0.8 µM; [17OH-PROG] = 2 – 32 µM; unfermented rooibos aqueous extract = [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

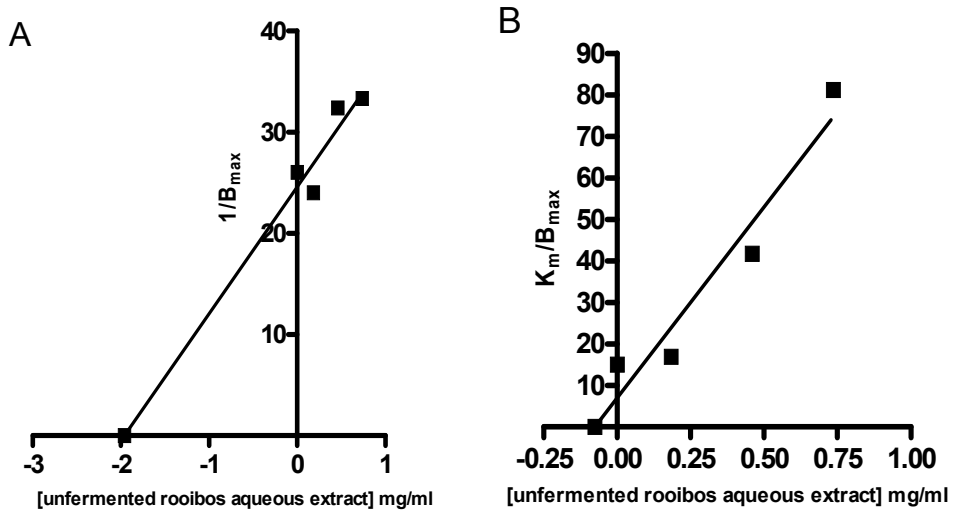


Figure 5.9. Secondary plots for mixed inhibition of 17OH-PROG binding to microsomal P450 enzymes in the presence of unfermented rooibos aqueous extract A) plot of $1/B^{app}_{max}$ against extract concentration and B) plot of K_m/ B^{app}_{max}. [P450] = 0.8 µM; [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

5.3.3 Microsomal conversion assays

The interactions of the rooibos extracts with the microsomal cytochrome P450 enzymes led to further investigations into the influence of extracts on the catalytic activity of these enzymes. In steroid hormone biosynthesis CYP17 is at a key branch point in the biosynthesis of cortisol, aldosterone and androgens. Inhibition of PREG or PROG metabolism by CYP17 would have an affect in the production of androgens and glucocorticoids such as cortisol. Inhibition of CYP21 activity would inhibit the production of the glucocorticoids and mineralocorticoids and shunt steroid biosynthesis in the direction of androgen production.

The influence of rooibos extracts on the catalytic activity of CYP17 was investigated by adding PREG to ovine adrenal microsomes in the presence of trilostane, a 3βHSD inhibitor. Although CYP17 catalyzes the conversion of PREG to 17OH-PREG and DHEA in most species, the lyase activity of ovine CYP17 for 17OH-PREG is not significant [Swart *et al*., 2003] as was observed when PREG was metabolized by ovine adrenal microsomes in the absence of rooibos extracts (figure 5.10). HPLC analysis indicated that 15 min after the initiation of the reaction 83 % of PREG was converted to 17OH-PREG, with no DHEA being detected. In the presence of methanol and aqueous extracts of unfermented as well as methanol extracts of fermented rooibos, the conversion of PREG was inhibited significantly ($P<0.001$). PREG conversion was inhibited 90 % and 88% by the methanol extracts of unfermented and fermented rooibos respectively, while the aqueous extract of unfermented rooibos exhibited the highest inhibition, 93 %. The aqueous extract of fermented rooibos showed a lower degree of inhibition ($P<0.01$), inhibiting PREG metabolism by 55 %. HPLC analysis of the steroid metabolites are illustrated n figure 5.11.

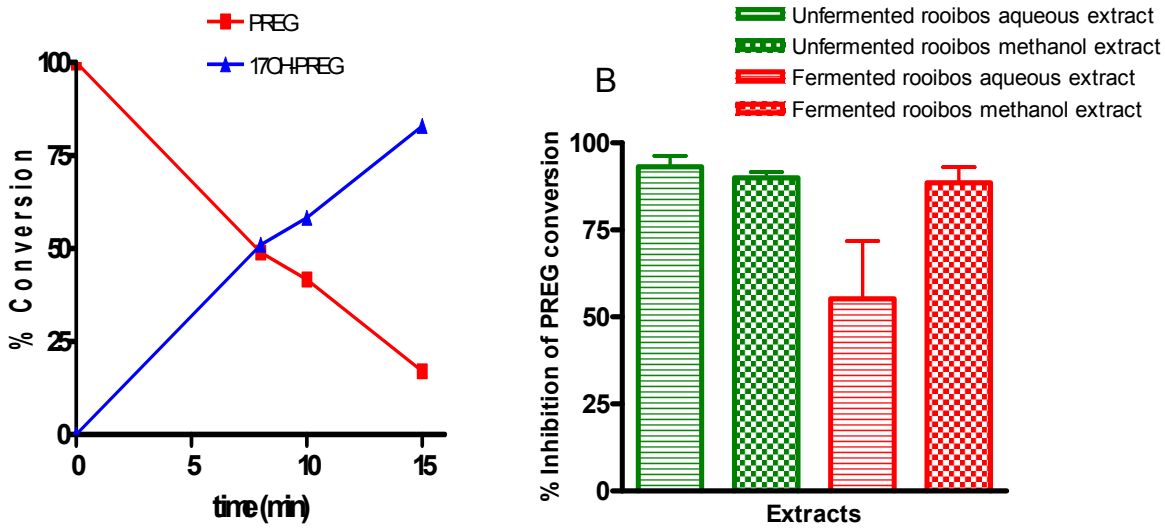


Figure 5.10. Metabolism of PREG (10 µM) by ovine adrenal microsomal P450 enzymes ([P450] = 0.35 µM) after initiation with NADPH (1 mM). (A) Percentage conversion in the absence of rooibos extracts and (B) percentage inhibition of PREG (after 15 min) in the presence of rooibos extracts. Unfermented rooibos: [aqueous extract] = 2.3 mg/ml, methanol extract = 2.3 mg/ml); fermented rooibos: [aqueous extract] = 2.3 mg/ml and methanol extract = 2.3 mg/ml. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PREG metabolism by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

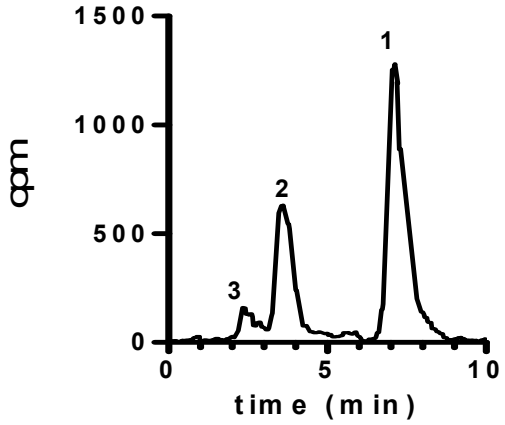


Figure 5.11. HPLC analysis of PREG (10 µM) metabolism by ovine adrenal microsomes at 10 min. Peak 1, PREG (7.5 min); peak 2, 17OH-PREG (3.5 min); and peak 3, DHEA (2.2 min).

In adrenal microsomes, the conversion of PROG to its subsequent metabolites is catalyzed by both CYP17 and CYP21. The conversion of PROG to 17OH-PROG is catalyzed by CYP17

whereas the conversion of PROG and 17OH-PROG to DOC and deoxycortisol, respectively, is catalyzed by CYP21. The conversion of PROG in the absence of rooibos extracts is summarized in figure 5.11 A. Approximately 99 % of PROG was converted to its metabolites, with 4.5 % 17OH-PROG, 71.85 % deoxycortisol and 21.74 % DOC being produced 15 min after initiation of the reaction.

In the presence of aqueous and methanol extracts of unfermented as well as methanol extracts of fermented rooibos inhibition was significant ($P< 0.01$) with PROG metabolism being inhibited by 83 %, 81 % and 79 %, respectively after 15 min of incubation (figure 5.12. B). Since the inhibition of PROG metabolism was high with the metabolites formed being negligible, analysing the steroid metabolities to determine the effect on CYP17 and CYP21 activity was hampered. In the presence of the aqueous extract of fermented rooibos, PROG metabolism was inhibited by 57.5 %. The formation of metabolites (figure 5.14) shows that the inhibition of the conversion of PROG by CYP21 in the presence of the aqueous extract of fermented rooibos to DOC is greater than the conversion of PROG to deoxycortisol. If 17OH-PROG accounts for the activity of CYP17, the data shows that that the inhibition of CYP21 is greater than the inhibition of CYP17. HPLC analysis of the steroid metabolites are illustrated n figure 5.13.

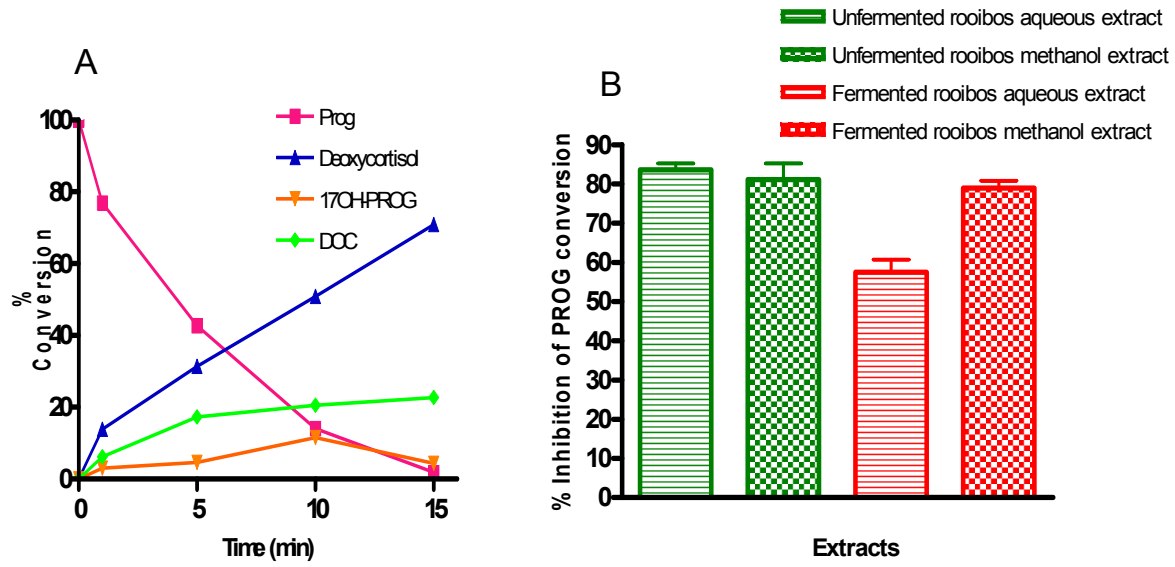


Figure 5.12. Metabolism of PROG (10 µM) by CYP17 and CYP21 ([P450] = 0.35 µM) in ovine adrenal microsomes after initiation with NADPH (1 mM) to deoxycortisol, DOC and 17OH-PROG metabolites. (A) Percentage conversion in the absence of rooibos extracts and (B) percentage inhibition of PROG conversion (after 15 min) in the presence of rooibos extracts. Unfermented rooibos: [aqueous extract] = 2.275 mg/ml, methanol extract = 2.25 mg/ml); fermented rooibos: [aqueous extract] = 2.3 mg/ml and methanol extract = 2.3 mg/ml. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG metabolism by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

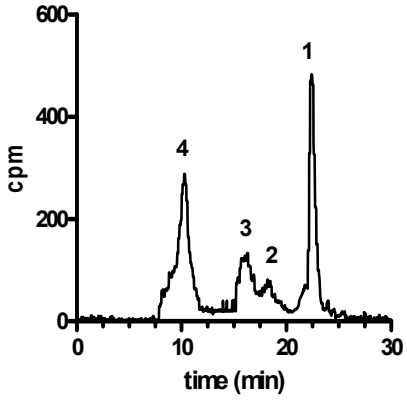


Figure 5 .13. HPLC analysis of PROG (10 µM) metabolism by ovine adrenal microsomes at 10 min. Peak 1, PROG (23 min); peak 2, 17-OH-PROG (19.5 min); peak 3, DOC (16.8 min); and peak 4, deoxycortisol (10 min).

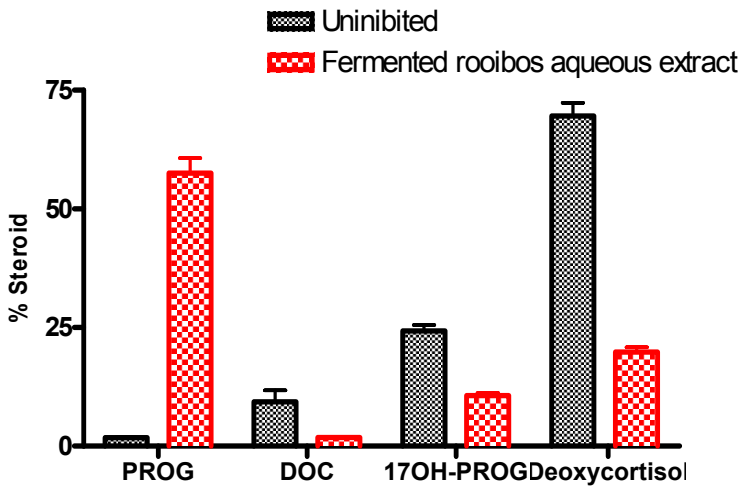


Figure 5.14. Conversion of PROG (10 µM) to steroid metabolites DOC, 17OH-PROG and deoxycortisol in the absence and presence of fermented rooibos aqueous extract in ovine adrenal microsomes [P450] = 0.35 µM) after initiation with NADPH (1 mM); [Fermented rooibos aqueous extract] = 2.3 mg/ml. Results are presented as the mean, error bars represent SEM and n=3.

5.3.4 COS1 conversion assay

The influence of rooibos extracts on the catalytic activity of the individual enzymes was investigated by expressing CYP17 and CYP21 in COS1 cells.

Baboon CYP17 expressed in COS1 cells, in the absence of rooibos extracts, converted 97 % PROG to 17OH-PROG and negligible amounts 4-androstenedione. In the presence of the unfermented rooibos methanol extract, the conversion of PROG to its metabolites was significantly inhibited ($P<0.001$) with 41 % being converted. PROG conversion in the presence of the fermented rooibos methanol extract was inhibited to a lesser degree, 39 % ($P<0.01$). Results are illustrated in figure 5.15 A.

In COS1 cells expressing baboon CYP21 70 % PROG was converted to DOC in the absence of rooibos extracts. Both unfermented and fermented rooibos methanol extracts inhibited the conversion of PROG to DOC significantly ($P<0.001$), with 30% DOC being formed (figure 5.15 B).

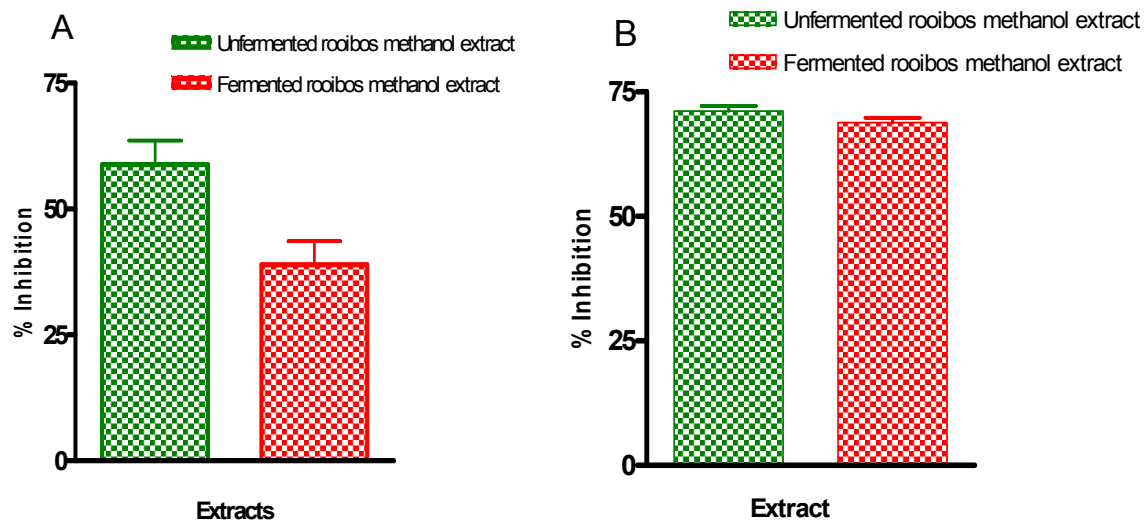


Figure 5.15. Percentage inhibition of PROG (1 µM) metabolism in COS1 cells expressing A) baboon CYP17 and B) baboon CYP 21 in the presence of methanol extracts of unfermented and fermented rooibos. [Unfermented rooibos methanol extract] = 4.5 mg/ml; [fermented rooibos methanol extract] = 4.6 mg/ml. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG metabolism by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

Steroid metabolites were analysed using LC-MS. The elution profile of PROG and DOC standards is shown in figure 5.16. Stock solutions of PROG and DOC (2 mg/ml ethanol) were used to prepare a series of standards, (2, 20, 200, and 2000 ng/ml) in methanol from the stock solutions. A standard curve was generated for each steroid with concentrations ranging from 10 to 1000 ng/ml. The calibration curves were linear over these concentration ranges (r^2 better than 0.99).

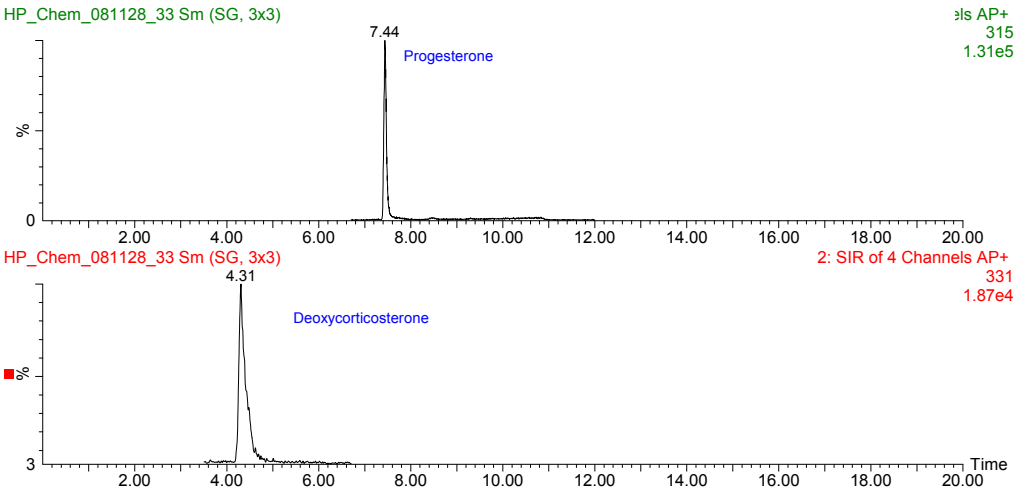


Figure 5.16. LC-MS analysis of PROG and DOC standards. PROG: 315 *m/z* retention time, 7.44 min and DOC: 331 *m/z* retention time, 4.32 min.

The influence of the rooibos extracts on the viability of COS1 cells was established to confirm that the steroid conversion would be as a result of the effect of the extracts and not due to cell death. The inherent ability of COS1 cells to convert testosterone to 4-androstenedione was used during the cell viability assay. There was no significant difference in the amount of 4-androstenedione produced by the cells incubated with rooibos extracts and the cells incubated without any extracts. The conversion of testosterone to 4-androstenedione by COS1 cells after 24 hr was 98%, while cells in the presence of unfermented rooibos methanol extract converted 96% of the testosterone assayed. The presence of the rooibos extracts thus does not have a toxic effect on the cells and a decrease in steroid conversion can be attributed to the influence of the extracts on CYP17 and CYP21.

5.3.5 HPLC Fractionation of an unfermented rooibos methanol extract

Based on the elution profiles of the aqueous and methanol extracts of unfermented rooibos and the bioactivity of the extracts being comparable, the methanol extract was fractionated using HPLC and five fractions were collected as shown in figure 5.17 based on their hydrophobicity.

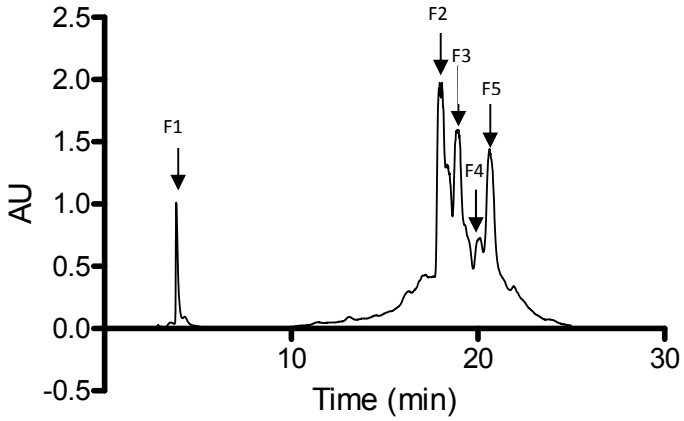


Figure 5.17. HPLC analysis of unfermented rooibos methanol exctract; fraction 1, F1; fraction 2, F2; fraction 3, F3; fraction 4, F4; fraction 5, F5.

5.3.6 Inhibition of P450 enzymes by unfermented rooibos methanol fractions

Substrate binding assay

The influence of the individual fractions, isolated from the unfermented rooibos methanol extract, on PROG binding to adrenal microsomal P450 enzymes was investigated. Fractions 3 and 5 exhibited a significant inhibitory effect with fraction 3 having the highest inhibitory effect, inhibiting PROG binding by ≈50 % ($P<0.01$), shown in figure 5.18. Fraction 5 inhibited PROG binding by ≈ 30 % ($P<0.05$). Fractions 1, 2 and 4 all inhibited PROG binding to a lesser degree, ≈15 % ($P>0.05$).

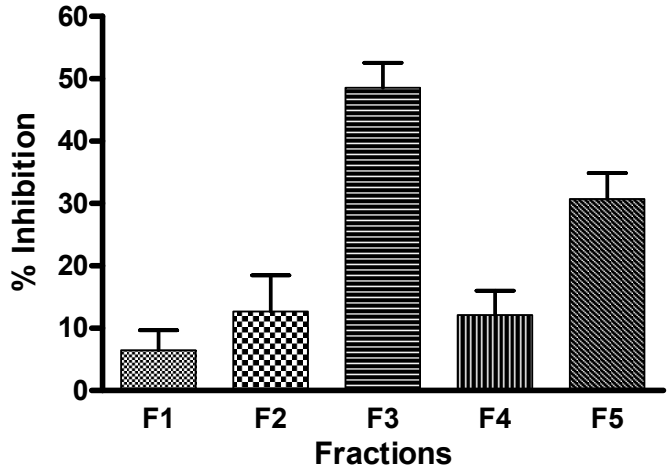


Figure 5.18. Percentage inhibition of PROG (16 µM) binding to ovine adrenal microsomal P450 enzymes ([P450] = 0.8 µM) by the individual unfermented rooibos methanol fractions. Fraction 1, F1; fraction 2, F2; fraction 3, F3; fraction 4, F4; fraction 5, F5. [Unfermented rooibos methanol fractions] = 0.08 mg/ml. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG metabolism by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

PROG conversion assay in COS1 cells

The influence of the five fractions on the conversion of PROG in COS 1 cells expressing CYP17 and CYP21 was assayed. None of the fractions inhibited the conversion of PROG to its subsequent metabolites in COS1 cells expressing CYP17. However, the catalytic activity of CYP21 was affected significantly with the conversion of PROG to DOC being inhibited by fractions 1, 2, 3, and 5 (figure 5.19). Fraction 1, 2 and 3 inhibited the conversion significantly ($P<0.001$) with fraction 2 displaying the highest degree of inhibition, 62.8 %, fraction 3, 56 % and fraction 1 43.6 % . Fraction 5 inhibited the conversion to a lesser degree, 33% ($P<0.01$).

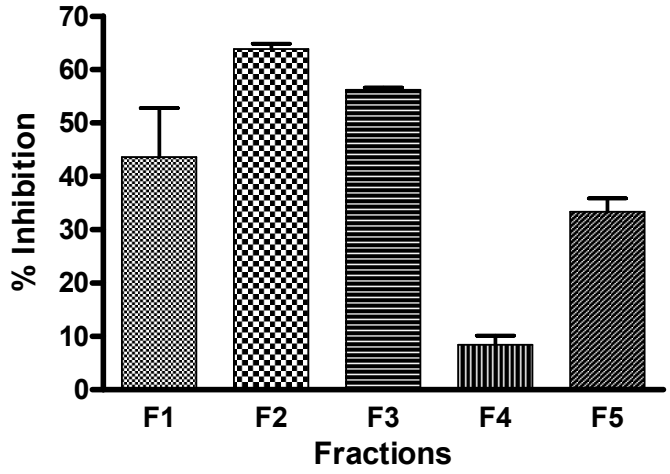
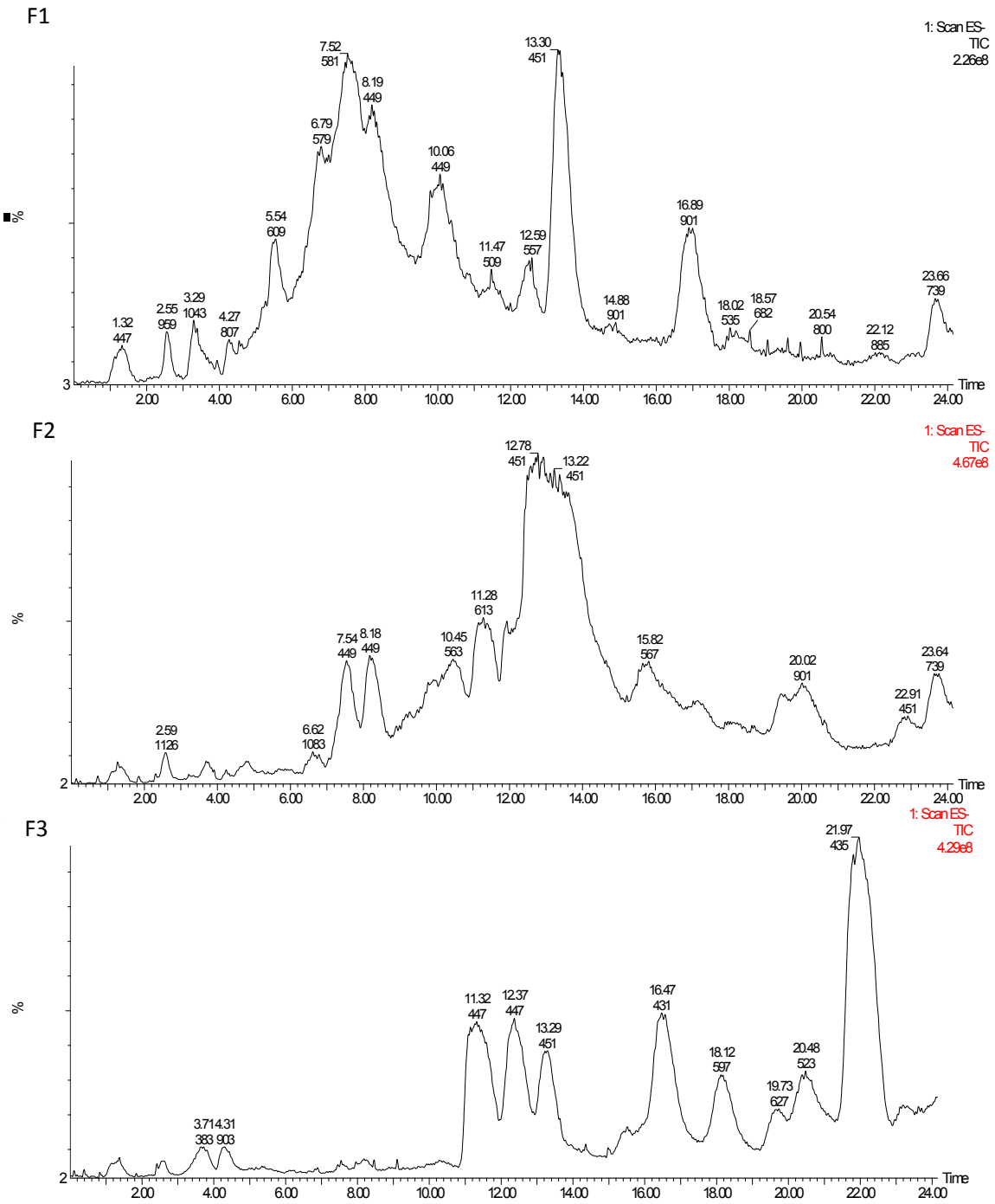


Figure 5.19. Percentage inhibition of PROG (1 μM) metabolism in COS1 cells expressing baboon CYP21 in the presence of unfermented rooibos methanol extract fractions. Fraction 1, F1; fraction 2, F2; fraction 3, F3; fraction 4, F4; fraction 5, F5. Unfermented rooibos methanol fractions] = 0.5 mg/ml Results are presented as the mean, error bars represent SEM and n=3.

5.3.7 LC-MS of bioactive fractions

The unfermented methanol fractions were subjected to LC-MS analysis to investigate the composition of each fraction. The chromatographic profile and molecular mass of each fraction (figure 5.20) was compared to the profiles and molecular masses of standard flavonoid compounds, vitexin, orientin, isoorientin, isovitexin and rutin which allowed the identification and the determination of the concentration of the compounds in the individual fractions (table 5.4). Fraction 2 and 3 which exhibited the highest degree of inhibition of substrate conversion in COS1 cells, contained the highest concentration of orientin. Fraction 3, which exhibited the highest degree of inhibition of PROG binding in microsomal preparations, also contained high concentrations of isoorientin and vitexin. None of the standard compounds were present in fraction 1. Rutin, which was only present in fraction 5, showed a significant degree of inhibition of substrate binding and conversion. Isovitexin was only detected in fraction 4 which exhibited no bioactivity with regards to inhibition of substrate binding or substrate conversion.


F1
1: Scan ES-
TIC
2.26e8
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3
Time
1.32 447
2.55 959
3.29 1043
4.27 807
5.54 609
6.79 579
7.52 581
8.19 449
10.06 449
11.47 509
12.59 557
13.30 451
14.88 901
16.89 901
18.02 535
18.57 682
20.54 800
22.12 885
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F2
1: Scan ES-
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2.59 1126
6.62 1083
7.54 449
8.18 449
10.45 563
11.28 613
12.78 451
13.22 451
15.82 567
20.02 901
22.91 451
23.64 739
F3
1: Scan ES-
TIC
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Time
3.71 383
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12.37 447
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20.48 523
21.97 435

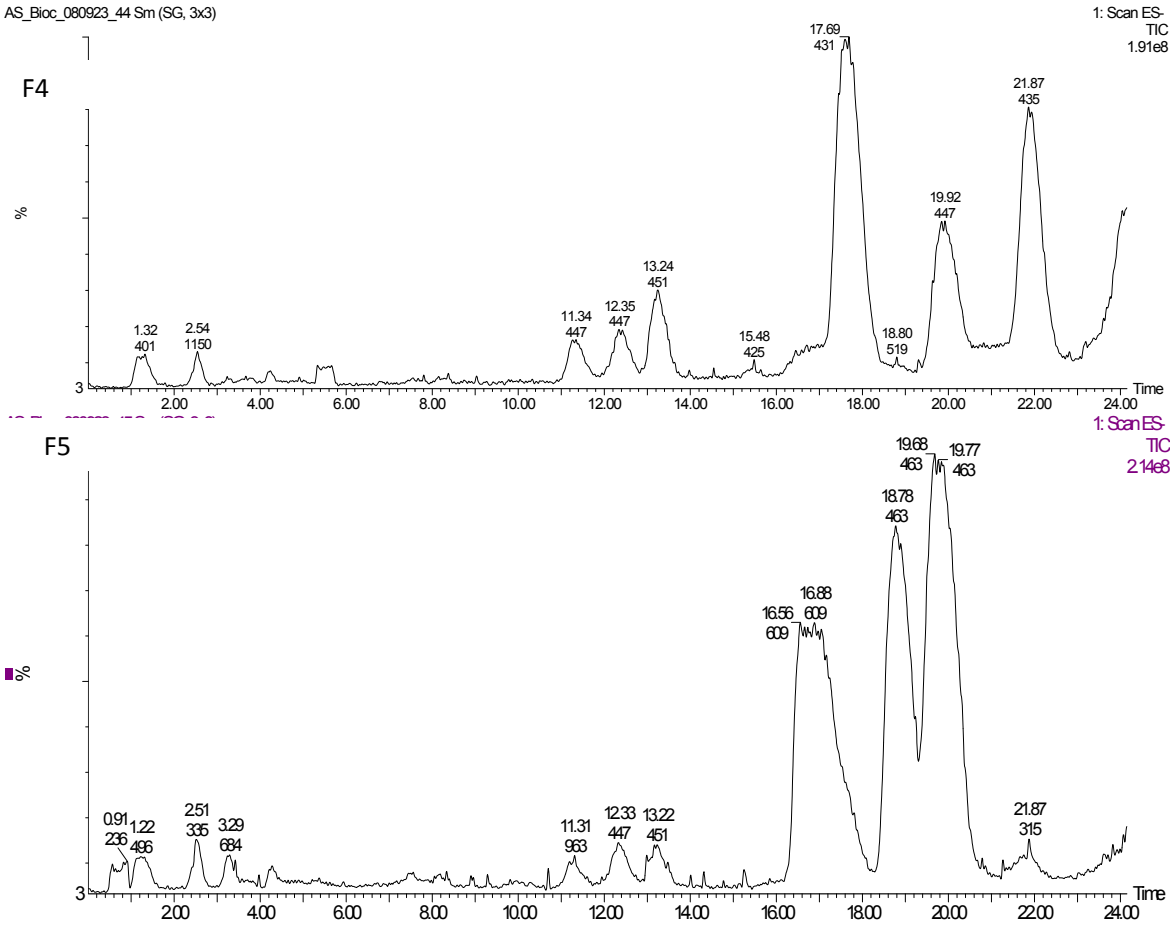


Figure 5.20. Total ion chromatogram (ESI negative) analysis of unfermented rooibos methanol fractions, fraction 1, F1; fraction 2, F2; fraction 3, F3; fraction 4, F4; fraction 5, F5.

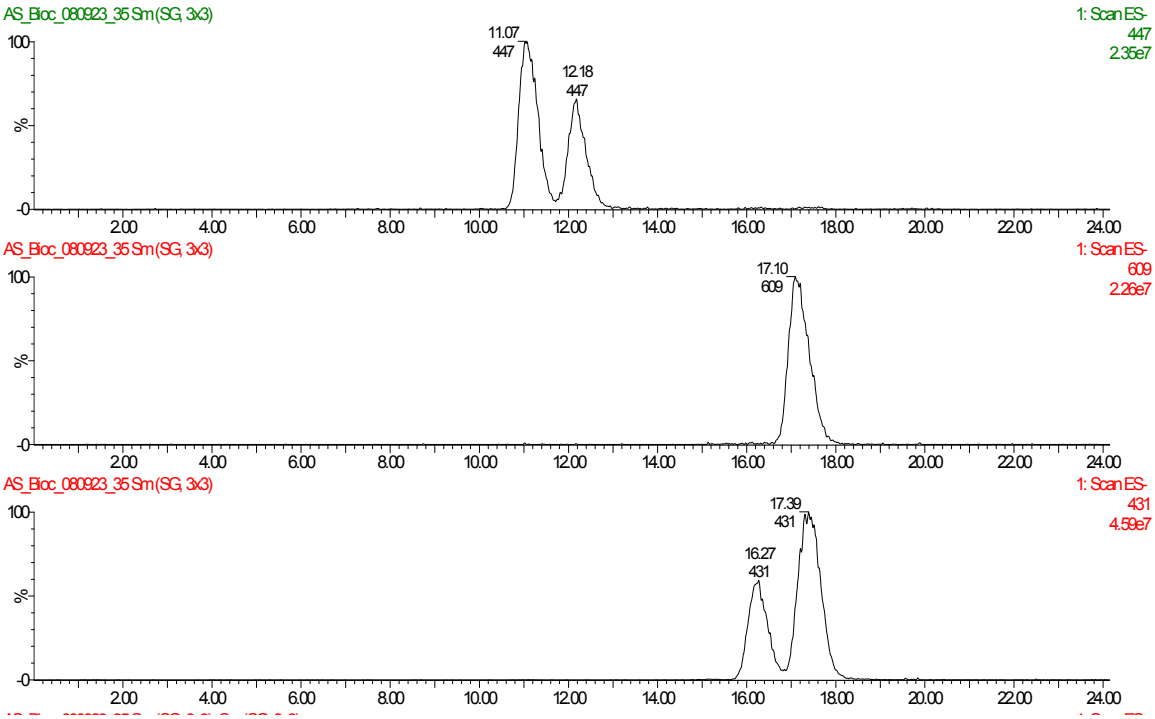


Figure 5.21. Total ion chromatogram (ESI negative) analysis of rooibos flavonoids. Iso-orientin: 447 *m/z* retention time, 11.03 min; orientin: 447 *m/z* retention time, 12.14 min; vitexin: 431 *m/z* retention time, 16.23 min; rutin: 609 *m/z* retention time, 17.09 min; isovitexin: 431 *m/z* retention time, 17.32 min.

Table 5.4: Concentration of standard flavonoid compounds (µg/ml) in unfermented rooibos methanol fractions

Compound	Fraction						
	Rt (min)	m/z	F1	F2	F3	F4	F5
Isoorientin	11.03	447	3.28	45.84	204.84	16.4	8.58
Orientin	12.14	447	4.6	149.82	179.02	21.99	17.64
Vitexin	16.23	431	2.1	15.77	140.55	5.59	2.86
Rutin	17.09	609	0.65	0	0	5.27	236.73
Isovitexin	17.32	431	0	7.68	0.69	110.91	11.13

5.3.8 Inhibition of P450 enzymes by rooibos flavonoid compounds

PROG conversion assay in COS1 cells

Analysis of the rooibos fractions revealed that rutin, vitexin and orientin were the main flavonoid compounds detected in the active fractions. Considering that the rooibos fractions only inhibited the catalytic activity of CYP21 and not CYP17, the influence of these flavonoid compounds on the catalytic activity of CYP21 was subsequently investigated. Previous assays carried out showed that isovitexin had no inhibitory effect on the P450 enzymes and was thus not included in the further investigations [Richfield R, 2007]. Due to structural similarity with isovitexin, iso-orientin was also not included in further bioactivity assays. Two different concentrations of each compound were used to determine if the compounds affect the catalytic activity of CYP21. Results revealed that a higher degree of inhibition is obtained with the final concentration of 10 μM.

The three compounds, rutin, vitexin and orientin, inhibited the CYP21 catalyzed conversion of PROG to its steroid metabolite, DOC, significantly ($P<0.001$). Orientin exhibited the highest degree of inhibition (47%), followed by vitexin (43%) and rutin (32%). Results are depicted in figure 5.22.

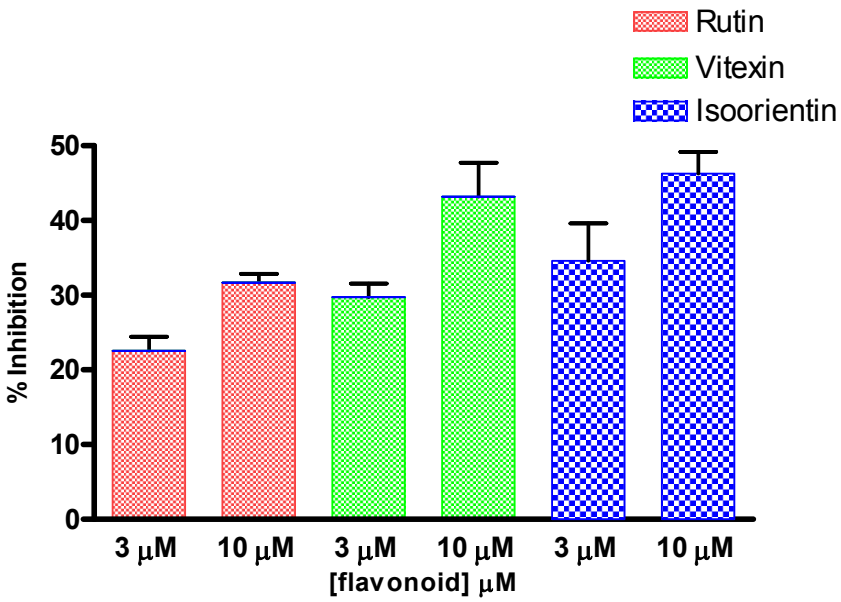


Figure 5.22. Percentage inhibition of PROG (1 μM) metabolism in COS1 cells expressing baboon CYP21 in the presence of rooibos flavonoid compounds rutin, vitexin and orientin. [Rutin] = 3 and 10 μM; [vitexin] = 3 and 10 μM; [orientin] = 3 and 10 μM. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG metabolism by the compounds was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($p < 0.001$).

5.4 Discussion

The bioactivity of unfermented and fermented rooibos extracts was investigated by determining the influence on P450 enzymes, CYP17 and CYP21. These enzymes are at a branch point in adrenal steroidogenesis and their inhibition would affect the outcome of adrenal steroidogenesis. The binding of endogenous substrate to the enzymes and subsequent substrate conversion were thus assayed. Once activity had been established, the methanol extract of unfermented rooibos was fractionated and individual fractions were assayed to determine the inhibition of the binding of substrates to CYP17 and CYP21 as well as the catalytic activity of these enzymes. The influence of flavonoid compounds present in the fractions on the catalytic activity of CYP21 was subsequently assayed.

The initial investigation into the influence of unfermented and fermented rooibos methanol and aqueous extracts on the binding of PROG to adrenal P450 enzymes revealed that unfermented rooibos inhibited the binding of PROG to a greater degree than fermented fractions, with the fermented aqueous extract having the weakest inhibitory effect ($P< 0.01$). It is therefore possible that compounds to which the inhibition of substrate binding to the adrenal microsomal P450 enzymes may be attributed are degraded during the oxidation process. As the unfermented rooibos aqueous extract had exhibited the highest degree of inhibition ($P<0.001$) the inhibitory effect of various concentrations of the extract on the binding of PROG and 17OH-PROG to microsomal P450 enzymes was subsequently assayed. The extract inhibited the binding of these substrates in a dose-dependent manner. The binding of PROG to microsomal P450 enzymes was competitively inhibited as the $K`_i$ and $K_i$ values remained the same and the $B_{max}$ value also remained unchanged in the presence of the extract. Unfermented rooibos aqueous extracts inhibited the binding of 17OH-PROG to microsomal P450 enzymes by mixed inhibition as the $K`_i$ and K_i values for 17OH-PROG binding differ. The inhibitor constant, K_i, which indicates the concentration required to produce half maximum binding inhibition, depicts the potency of the inhibitor. The K_i value of 17OH-PROG (0.08 mg/ml) is lower than the K_i value for PROG (1.3 mg ml) indicating that a higher concentration of extract is required to inhibit the binding of 17OH-PROG.

Substrate conversion assays revealed that the catalytic activity of CYP17 and CYP21 was indeed influenced by the presence of rooibos extracts as the metabolism of both PREG and PROG in adrenal microsomes was inhibited. Unfermented rooibos aqueous and methanol extracts showed a stronger inhibitory influence on PREG and PROG metabolism than the fermented extracts. Fermented rooibos aqueous extract again showed the weakest inhibitory influence. Analysis of the formation of PROG steroid metabolites indicates that the fermented rooibos aqueous extract has a stronger inhibitory effect on the catalytic activity of CYP21 than CYP17.

Since adrenal microsomes contain both CYP17 and CYP21, the effect of the rooibos extracts on the individual enzymes was investigated by expressing the enzymes in COS1 cells. Both unfermented and fermented rooibos methanol extracts inhibited the conversion of PROG by

CYP17 significantly ($P<0.001$) with the unfermented rooibos methanol extract having a stronger inhibitory effect on the catalytic activity of CYP17 (>58%) than the fermented extract (>38%). Both the unfermented and fermented rooibos methanol fractions influenced the catalytic activity of CYP21 significantly ($P<0.001$) by inhibiting PROG conversion by 70%. These data indicate, as data obtained using microsomal preparations, that the inhibitory effect of rooibos is greater with regards to CYP21. Since the extracts alone had no effect on cell viability the influence on the conversion of steroid substrates in the presence of rooibos extracts could thus be attributed to the inhibitory influence on the catalyitc activity of the P450 enzymes.

In view of the fact that the unfermented rooibos methanol extract significantly inhibited the binding and catalytic activity of microsomal P450 enzymes ($P<0.001$) and additional compounds were observed with LCMS analysis, the extract was fractionated in an attempt to identify specific compounds interacting with P450 enzymes. Substrate inhibition assays using PROG as substrate showed that fraction 3 exhibited the strongest inhibitory effect ($P<0.01$) on the binding of the substrate to adrenal microsomal P450 enzymes. The other fractions did not exhibit a marked influence on the binding of PROG, with the exception of the inhibition of fraction 5 ($P<0.05$).

The catalytic activity of CYP17 and CYP21 were also assayed in their presence. PROG conversion in COS1 cells expressing CYP21 was inhibited significantly with fractions 1, 2 and 3 ($P<0.001$) being the most potent and fraction 5 ($P<0.01$) inhibiting to a lesser degree. None of the fractions influenced the catalytic activity of CYP17 as there was no conversion of PROG in COS1 cells expressing the enzymes in the presence of the fractions. The inhibition of PROG binding by fraction 3 and 5 in microsomal preparations must therefore be attributed to the interaction of compounds in these fractions with CYP21.

LC-MS analysis of the unfermented rooibos methanol fractions was conducted to elucidate the composition of the fractions. Flavonoid compounds were detected by comparing their retention times and UV spectra to those of available standards. LC-MS analyses showed that fractions 2 and 3 have high concentrations of orientin, while fraction 3 also contains high levels of

isoorientin and is the only fraction that contains a significant amount of vitexin. Fraction 3 inhibited both the binding of PROG to adrenal microsomal P450 enzymes and the conversion of PROG by CYP21, indicating that vitexin, orientin and isoorientin could be the compounds responsible for the inhibitory effects observed. Fraction 5 also showed a significant degree of inhibition and LC-MS analyses revealed rutin to be the main compound present in the fraction. LCMS analysis also revealed that fraction 2 posibly has a high concentration of aspalathin, Mr 451, whereas fraction 3 may have a high concentration of nothofagin, Mr 435. This could not be confirmed due to the unavailability of standard compounds.

Previous binding inhibition studies [Richfield, 2007] carried out with methanol and chloroform extracts of fermented and unfermented rooibos showed that while the extracts all exhibited both competitive and non-competitive inhibition of PROG no competitive inhibition was observed in the case of 17OH-PROG, except in the case of the chloroform extract of fermented rooibos. The influence of C-glycosylflavones vitexin, orientin and rutin on the catalytic activity of CYP21 was investigated as these compounds were the most abundant in the active fractions 2, 3 and 5. The effect of the compounds on the catalytic activity of CYP17 was not investigated as none of the individual fractions had shown any influence on the enzyme. Results showed that orientin and vitexin had the strongest inhibitory influence on the catalytic activity of CYP21 with orientin (47%) exhibiting a slightly stronger effect than vitexin (43%).

In summary, CYP17 and CYP21 play a key role in the steroidogenesis pathway and the inhibition of these enzymes will result in an alteration or reduction of glucocorticoid levels. The study found that CYP17 is inhibited by rooibos extracts whereas, once fractionated, activity towards CYP17 is lost. Rooibos and flavonoids present may interact with the P450 enzymes thereby reducing cortisol levels by inhibiting key steroidogenic enzymes and providing preliminary evidence for the reported stress relieving properties of rooibos tea.

Chapter 6

Conclusion

Africa, especially southern Africa, has a diverse variety of plants with about 3000 medicinal plants identified to date. It has generally been established that the use of natural products will play a major role in the socioeconomic development in Africa. The diversity of medicinal plants provides a valuable platform, not only for commercial development, but also for basic scientific study [Van Wyk, 2008]. Even in modern times, many traditional cultures still remain largely dependant on plants for their food and medicinal needs and approximately 80% of the global population use plant extracts for their primary health needs [Zhang, 2002]. The use of herbal preparations as medicinal remedies is becoming increasingly popular and healthcare providers in Europe and Asia often prescribe herbal teas. Rooibos is a well known indigenous herbal tea and is believed to have medicinal properties. However, the therapeutic uses and apparent health benefits of rooibos are, like most herbal preparations, based on folklore rather than on scientific evidence [Huffman, 2003].

The international demand for rooibos has gradually grown from 750 tons in 1993 to 7200 tons in 2007. Rooibos has gained popularity due to its fruity, sweet taste and its caffeine-free, low tannin, antioxidant-rich status. Anecdotal evidence suggests that rooibos tea has anti-allergenic effects, reduces nervous tension, calms digestive disorders and various stomach problems. Rooibos tea also exhibits anti-depressive properties which counteract nervous tension and anxiety and aids sleeplessness [Joubert *et al*., 2008]. These conditions are linked to a dysfunctional endocrine system characterised by abnormal cortisol levels, thereby justifying an investigation of the influence of rooibos on the endocrine system.

Three of the major glands that make up the human endocrine system include the hypothalamus, pituitary and adrenal glands. The HPA axis is activated in response to a stressor and cortisol secretion by the adrenal gland is stimulated. Glucocorticoids play an important role in the control

of homeostasis during stress by prioritizing and redistributing energy as well as having a complex influence on mood and cognition. These effects demand strict control of glucocorticoid secretion not only to ensure an effective stress response but also to minimize the harmful effects of glucocorticoid excess. During chronic stress, the inhibitory cortisol feedback, that acts to limit the duration of the total tissue exposure to cortisol, is uplifted and the ensuing catabolic, anti-reproductive and immunosuppressive effects of this hormone can lead to various diseases [Tsigos and Chrousos, 2002; Jacobson., 2006].

During the biosynthesis of glucocorticoids in the adrenal gland, the hydroxylation and cleavage of the steroid precursor substrates is catalyzed by P450 enzymes. P450 enzymes act as monooxygenases in the reaction by utilizing reduced NADPH as the electron donor for the reduction of molecular oxygen. Two separate electron transfer systems are responsible for the transfer of electrons from NADPH to the substrate. Mitochondrial P450 enzymes receive electrons from NADPH via NADPH-ADXR and ADX and finally deliver the electrons to the substrate. Only one protein is involved in the microsomal electron transfer, cytochrome P450 oxidoreductase which contains two flavins. Electrons are sequentially transferred from NADPH to FAD followed by transfer to FMN, P450 and the substrate.

The relative activities of the microsomal P450 enzymes, CYP17 and CYP21, determine the flux of steroid intermediates in the adrenal steroidogenic pathway. Inhibition of CYP21 would result in an increase in the flux towards androgen synthesis whereas activation would direct metabolism towards the synthesis of glucocorticoids and mineralocorticoids. The catalytic site of P450 enzymes contains a heme prosthetic group and the iron protoporphyrin IX complex is bound in part by hydrophobic forces in the active site of P450 enzymes. The axial heme iron ligands mainly determine the specific function of the hemoproteins and the characteristic thiolate ligand of the P450 enzymes plays role in the unusual spectral and catalytic properties of these enzymes. The complex nature of the catalytic site of the P450s makes it vulnerable to inhibition by compounds in different ways – inhibitory compounds can bind to the active site and induce a conformational change that prevents the binding of natural substrates, compounds can bind to a

site different than the active site and interfere with the binding of natural substrates and electron transport proteins, or they could also bind to the enzyme-inhibtor complex

The influence of unfermented and fermented rooibos extracts on the endocrine system was investigated by determining its influence on the adrenal steroidogenesis P450 enzymes, CYP17 and CYP21. Spectral assays revealed that the type I difference spectra produced by PROG binding to adrenal microsomal P450 enzymes were inhibited in the presence of rooibos extracts. Unfermented rooibos methanol and aqueous extracts had a stronger inhibitory influence than the fermented extracts, suggesting that bioactive compounds, degraded during the fermentation process, could play an important role in the inhibition of substrate binding.

Unfermented rooibos aqueous extract exhibited the strongest binding inhibition and subsequent spectral assays demonstrated that the extract inhibits the binding of PROG and 17OH-PROG in a dose dependent manner. As can be seen from the data obtained in the primary plots the interaction of these substrates with the microsomal enzymes differs markedly. This may be due to the fact that the microsomal preparation contains both CYP17 and CYP21 enzymes and while PROG and 17OH-PROG are both natural substrates for CYP21, CYP17 catalyses only the conversion of PROG and 17OH-PROG is not a substrate for CYP17 in adrenal steroidogenesis. The binding of 17OH-PROG may possibly not be influenced as much as the binding of PROG at lower concentrations of the extracts. To characterize the inhibition and to determine the inhibition constant (K_i), the data were analyzed using double reciprocal and secondary plots. These plots revealed that the binding of PROG, in the presence of unfermented rooibos aqueous extract, was inhibited competitively. Competitive inhibitors compete with the substrate for the active site of the enzyme and have the affect of increasing the K_m of the reaction and therefore reduce the affinity of the enzyme for its substrate. It would seem that 17OH-PROG is inhibited by mixed inhibition, as indicated by the $K`_i$ and K_i values for the binding of 17OH-PROG in the presence of unfermented rooibos aqueous extracts. Mixed inhibitors have the affect of partly reducing the affinity of the enzyme for the substrate and partly reducing the maximum reaction rate.

The bioactivity of unfermented and fermented rooibos extracts was investigated by determining the influence on the catalytic activity of P450 enzymes, CYP17 and CYP21. *In vitro* metabolic assays demonstrated that unfermented and fermented rooibos extracts inhibit the conversion of steroid substrates to their subsequent metabolites by inhibiting adrenal microsomal P450 enzymes. Unfermented rooibos methanol and aqueous extracts again exhibited a stronger inhibitory influence than the fermented extracts.

The greater inhibition shown by unfermented rooibos extracts in comparison to fermented extracts could be ascribed to the degradation of compounds during the fermentation process. Fermented rooibos extracts did, however, exhibit a high degree of inhibition, indicating that other compounds that are more stabile could be responsible for the inhibitory effect of fermented rooibos. Aspalathin is oxidized to dihydro-iso-orientin and dihydro-orientin during fermentation, with isoorientin and orientin degraded to a lesser degree. Rutin is partly converted to the aglycone, quercetin. Prominent peaks with high molecular masses were observed in the unfermented methanol extract. It is possible that these compounds may exhibit activity towards the P450 enzymes, not competing directly with the substrate for the active site due to their size, but perhaps binding to the enzyme-substrate complex. The influence of the unfermented and fermented methanol extracts on the catalytic activity of CYP17 and CYP21 in COS1 cells were subsequently investigated, showing the inhibition of the catalytic activity of CYP21 to be significantly greater than the inhibition of CYP17. In addition, the unfermented rooibos methanol extract had a stronger inhibitory effect on the catalytic activity of CYP17 than the fermented extract. Earlier studies [Richfield, 2007] showed that the binding of PREG was not inhibited by rooibos while this study showed clearly that PREG metabolism is inhibited. Interestingly, upon purification and fractionation of the extract this activity is lost – indicating the presence of labile bioactive compounds in the plant responsible for the inhibition of PREG metabolism. These high molecular mass compounds were not observed upon fractionation of the unfermented rooibos methanol extract.

Once activity had been established, the methanol extract of unfermented rooibos was fractionated to ascertain whether specific compounds interacting with P450 enzymes could be

identified. The effect of five fractions on the binding of substrates to CYP17 and CYP21, as well as the catalytic activity of these enzymes expressed in COS1 cells was determined. Only two fractions, fractions 3 and 5, inhibited the binding of substrate to adrenal P450 enzymes significantly. The catalytic activity of CYP21 in COS1 cells was significantly inhibited by all the fractions with the exception of fraction 4. The fractions, however, had no inhibitory effect on the catalytic activity of CYP17. It is possible therefore that the inhibition observed using whole extract assays, as mentioned above, is as a result of the presence of very labile compounds that could have been chemically altered during the fractionation of the unfermented rooibos methanol extract.

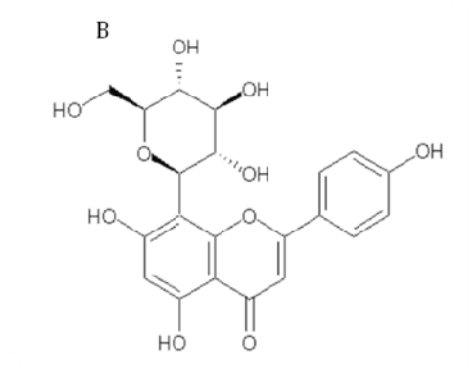
LCMS analysis data of the unfermented rooibos methanol fractions were conducted and flavonoid compounds were detected by comparing retention times and UV spectra with those of available standards. The three compounds that were the most abundant in the active fractions, rutin, vitexin and orientin, were subjected to a COS1 metabolic assay to determine their influence on the catalytic activity of CYP21. The flavonoids significantly inhibited CYP21 with orientin (10 μM) exhibiting the strongest inhibitory effect on the catalytic activity of CYP21, followed by vitexin and rutin. These findings correlate well with studies conducted by Ohno *et al*, 2002 in which flavonoid phytochemicals were shown to inhibit cortisol production in human adrenocortical H295R cells as well as inhibiting P450 enzymes. Upon exposure to concentrations ranging from 1 – 25 μM, CYP11A1 was not inhibited by any of the standard compounds. Significant inhibition of CYP17 ($P<0.05$) was seen only at 25 μM of the 6OH-flavone, while it inhibited CYP21 ($P<0.01$) at 12.5 μM. Daidzein (3 μM) and genistein (1 μM) exhibited no inhibition towards CYP17 or CYP21 with only CYP21 being inhibited ($P<0.01$) by both compounds at 12.5 μM [Ohno *et al*., 2002].

Flavonoids present in rooibos share a structural homology with steroid substrates and could therefore bind in the active site of the P450 enzyme and hamper the binding of natural steroid substrates. These inhibitory compounds that are structurally related to the steroid substrates, could thus cause a shift in the spin equilibria of the substrate bound cytochrome P450 enzymes. The flavonoid and extracted compounds may be smaller than the natural substrate and thus have

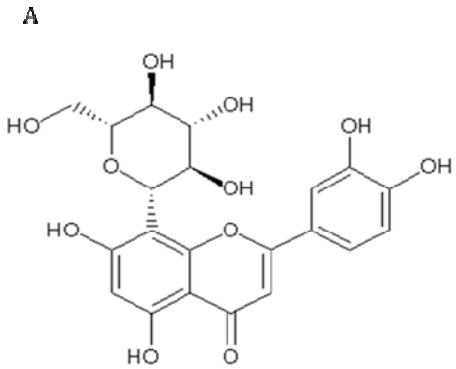
greater freedom of movement within the active site. Crystal structure studies of complexes of substrate analogues with P450cam have shown that the Fe^{3+}- coordinated water molecule is retained and a high fraction of the low spin character of the Fe^{3+} remains [Segall *et al*., 1998]. The spin equilibria of substrate analogue-bound enzymes thus favor the low spin state. The redox potentials of the substrate analogue-bound complexes fall between those of the substrate-free and substrate-bound systems, thus the reduction of the P450 is energetically less favorable and the catalytic reaction could be inhibited by the active compounds present in rooibos. The compounds may also inhibit substrate binding by binding to a site other than the active pocket; thereby causing structural changes in the enzyme which subsequently hampers the binding of the natural substrate. Further structural studies have to be conducted to elucidate the exact nature of interaction between the compounds and the P450 enzymes.

Structural studies of flavonoids have shown that the only difference between orientin (structure A) and vitexin (structure B) is at the 3′ position of the benzene ring. Orientin and vitexin are both glycosylated at position 8 of the pyran ring, in contrast to rutin (structure C) which is glycosylated at position 3. In the studies carried out by Ohno *et al* (2002), it was suggested that flavonoid compounds with a hydroxy group at positions 6 and 4′ appear to inhibit the catalytic activity of enzymes in the cortisol biosynthesis pathway. Both iso-orientin and iso-vitexin are glycosylated at position 6. Our data shows iso-orientin and iso-vitexin in fraction 3 and 4 respectively. Fraction 4 exhibited negligible inhibitory activity, and this may therefore be attributed to the large substitution at position 6. The iso-orientin present in fraction 3 possibly does not contribute towards the inhibitory activity of this fraction. It thus appears that the glycosylation at position 6 plays a role in the loss of inhibition activity of the flavonoids. However, this will need to be validated by correlating the structure of various compounds with their inhibitory potency as only three compounds were compared in this study. Furthermore, the inhibitory potency can be influenced by several aspects such as metabolism by other enzymes in the preparations, membrane transport mechanisms and the solubility of the flavonoids, all of which should be taken into consideration. A previous study has, however, shown that an increase of inhibitory potency of 17β-HSD is observed with the increasing degree of hydroxylation of

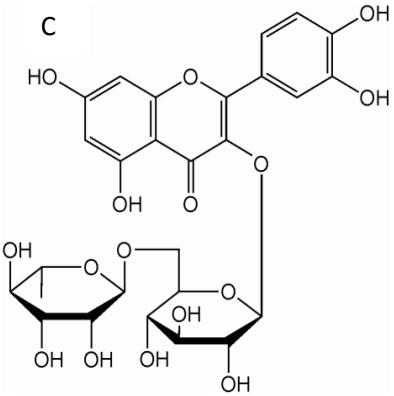
flavonoids [Krazeisen *et al*., 2001]. This indicates that these substances may exert their inhibitory effect by binding to the hydrophilic regions of the binding site of receptors enzymes.



vitexin



orientin



rutin

LCMS analyses of the extracts also revealed the possible presence of high levels of aspalathin, Mr 451 and nothofagin, Mr 435 in fractions 2 and 3, respectively. Unfermented rooibos is characterized by high concentrations of aspalathin and nothofagin which are degraded during the fermentation process [Joubert *et al*., 2005]. In a study conducted in our laboratory, quantitative analysis of polyphenols in rooibos showed aspalathin in unfermented rooibos 10.9% and in fermented 0.53%, with nothofagin in unfermented rooibos 1.26% and in fermented 0.11% [Richfield, 2007]. Although nothofagin has been shown to exhibit phytoestrogenic activity [Shimamura *et al*., 2006] it is also possible that aspalathin and nothofagin may interact with the P450 enzymes.

In summary, this study provides preliminary scientific evidence of stress relieving biochemical properties of rooibos tea. The results show that rooibos derived extracts and compounds inhibit adrenal steroidogenic enzymes, CYP17 and CYP21. Inhibition of these enzymes during chronic stress could result in a reduction of glucocorticoid secretion and protection against the detrimental effects of prolonged glucocorticoid secretion. The biosynthesis of cortisol is influenced by the catalytic activity of CYP17 and CYP21. Inhibition of these enzymes would result in the increase of flux towards the synthesis of androgens and a decrease in the flux towards cortisol synthesis. The structure of rooibos flavonoids is similar to that of the P450 substrates indicating their ability to bind and inhibit the catalytic activity of these enzymes. This study has shown for the first time that only CYP21 is inhibited by these compounds, especially orientin and vitexin. This data therefore clearly indicate that rooibos can exert a significant effect on the endocrine system via interaction with specific adrenal P450 enzymes.

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