THE INHIBITION OF ADRENAL STEROIDOGENIC ENZYMES AND MODULATION OF GLUCOCORTICOID LEVELS *IN VITRO* AND *IN VIVO* BY *ASPALATHUS LINEARIS* (ROOIBOS)

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly stated otherwise) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

This study describes:

- the influence of a methanolic extract of unfermented Rooibos and five major Rooibos flavonoids, aspalathin, nothofagin, rutin, orientin and vitexin, on the activities of key adrenal steroidogenic enzymes - cytochrome P450 17β-hydroxylase/17,20-lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase (3βHSD2), cytochrome P450 21-hydroxylase (CYP21A2) and cytochrome P450 11β-hydroxylase (CYP11B1), expressed in non-steroidogenic COS-1 cells;

- the development of a novel UPLC-MS/MS method for the separation and quantification of 21 adrenal steroid metabolites;

- the influence of Rooibos and aforementioned flavonoids on adrenal steroid hormone production in H295R cells - a human adrenal carcinoma cell line expressing the enzymes catalysing the production of mineralocorticoids, glucocorticoids and adrenal androgens, assayed under both basal (normal) and forskolin-stimulated (stressed) conditions;

- the influence of Rooibos on the inter-conversion between cortisol and cortisone by 11βHSD1 and 11βHSD2 expressed in CHO-K1 cells;

- the influence of Rooibos consumption on circulating steroid hormone levels and ratios in male Wistar rats;

- the influence of Rooibos consumption on circulating steroid hormone levels and ratios in male and female human test subjects at risk for developing cardiovascular disease.

OPSOMMING

Hierdie studie beskryf:

- die invloed van metanoliese ekstrakte van ongefermenteerde Rooibos en vyf van die hoof flavonoïedverbindings in Rooibos, aspalatien, notofagien, rutien, oriëntien en viteksien, op die aktiwiteite van ensieme wat steroïedbiosintese in die bynier kataliseer – sitochroom P450 17α-hidroksilase/17,20-liase (CYP17A1), 3β-hidroksisteroïed dehidrogenase (3βHSD2), sitochroom P450 21-hidroksilase (CYP21A2) en sitochroom P450 11β-hidroksilase (CYP11B1), uitgedruk in nie-steroïed produserende COS-1 selle;

- die ontwikkeling van ‘n geskikte UPLC-MS/MS metode vir die skeiding en kwantifisering van 21 steroïedmetaboliete in die bynier;

- die invloed van Rooibos en die bg. flavonoïede op steroïedproduksie in H295R selle – ‘n menslike bynier kanker sellyn gekenmerk deur die ekspressie van die steroidogeniese ensieme wat die produksie van mineralokortikoïede, glukokortikoïede en bynierandrogene kataliseer, ge-analiseer onder beide basale (normale) en forskoliengestimuleerde (gestresde) kondisies;

- die invloed van Rooibos op die omeenskakeling tussen kortisol en kortisoon deur 11βHSD1 and 11βHSD2 in CHO-K1 selle;

- die invloed van Rooibosinname op vlakke van sirkulerende steroïed hormone en relatiewe verhoudings in die bloed van manlike Wistarrotte;

- die invloed van Rooibosinname op sirkulerende steroïed hormoon vlakke en relatiewe verhoudings in die bloed van mans en vrouens met ‘n hoë risiko vir die ontwikkeling van kardiovaskulêre siektes.

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ABBREVIATIONS

11-DHC	11-dehydrocorticosterone
11KA4	11-ketoandrostenedione
11KT	11-ketotestosterone
11OHA4	11β-hydroxyandrostenedione
11OHT	11β-hydroxytestosterone
11βHSD1	11β-hydroxysteroid dehydrogenase type 1
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
16OH-PROG	16OH-progesterone
17OH-PREG	17OH-pregnenolone
17OH-PROG	17OH-progesterone
17βHSD	17β-hydroxysteroid dehydrogenase
18OH-CORT	18OH-corticosterone
3βHSD	3β-hydroxysteroid dehydrogenase
A4	androstenedione
ACAT	acyl-coenzyme A:cholesterol acyltransferase
ACE	angiotensin-converting enzyme
ACTH	adrenocorticotropic hormone
ALDO	aldosterone
AMPK	AMP-activated protein kinase
Ang I	angiotensin I
Ang II	Angiotensin II
AVP	arginine vasopressin
CAH	congenital adrenal hyperplasia
CBG	corticosteroid binding globulin
CNS	central nervous system
COMT	catechol-*O*-methyltransferase
CORT	corticosterone
CRH	corticotropin-releasing hormone
CVD	cardiovascular disease
CYP11A1	cytochrome P450 side-chain cleavage

CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17A1	cytochrome P450 17β-hydroxylase/17,20 lyase
CYP19	cytochrome P450 aromatase
CYP21A2	cytochrome P450 21-hydroxylase
Cyt-b_5	cytochrome b_5
DCM	diabetic cardiomyopathy
DHEA	dehydroepiandrosterone
DOC	deoxycorticosterone
ER	estrogen receptor
FAD	flavinadenine dinucleotide
FFA	free fatty acid
FMN	flavinmononucleotide
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
G6PDH	glucose-6-phosphate dehydrogenase
GI tract	gastrointestinal tract
GLUT	glucose transporter
GR	glucocorticoid receptor
GSH	Glutathione
H6PDH	hexose-6-phosphate dehydrogenase
HDL	high density lipoprotein
HPA axis	hypothalamic-pituitary-adrenal axis
HSD	hydroxysteroid dehydrogenases
HSL	hormone-sensitive lipase
IL-10	interleukin-10
IL- 6	interleukin-6
IMM	inner mitochondrial membrane
LBD	ligand binding domain
LDL	low density lipoprotein
LPH	lactase-phlorizin hydrolase
LPS	lipopolysaccharide
MetS	metabolic syndrome
MR	mineralocorticoid receptor

OMM	outer mitochondrial membrane
P450	cytochrome P450
PEPCK	phosphoenolpyruvate carboxykinase
POR	P450 oxidoreductase
PPAG	phenylpropenoic acid glucoside
PREG	pregnenolone
PROG	progesterone
RAAS	rennin-angiotensin aldosterone system
RNS	reactive nitrogen species
ROS	reactive oxygen species
SRBI	scavenger receptor class B, type-I receptor
StAR	steroidogenic acute regulatory protein
STZ	streptozotocin
SULT	sulfotransferase
T1D	type 1 diabetes
T2D	type 2 diabetes
TAC	total antioxidant capacity
TG	triglyceride
TNF-α	tumor necrosis factor-α
UGT	uridine-5’diphosphate glucuronosyl-transferase
WT	wild type

CHAPTER 1

General Introduction

Rooibos (*Aspalathus linearis*), a fynbos plant unique to the Western Cape region of South Africa, has traditionally been used for the alleviation of anxiety, sleeplessness and nervous tension – ailments generally associated with stress and abnormal glucocorticoid levels. Rooibos is a rich source of dietary polyphenols and while exhibiting potent anti-oxidant, anti-diabetic and cardio-protective properties, numerous *in vivo* studies have reported that its consumption also improves glucose homeostasis, insulin resistance and lipid profiles significantly. (Mose Larsen et al. 2008; Marnewick et al. 2011; Son et al. 2013; Ajuwon et al. 2014; Dludla et al. 2014). These clinical conditions are generally associated with abnormal glucocorticoid levels. Glucocorticoids are synthesized in the adrenal cortex by P450 11β-hydroxylase (CYP11B1), which catalyzes the conversion of deoxycorticosterone (DOC) and deoxycortisol to corticosterone (CORT) and cortisol, respectively. Glucocorticoid biosynthesis in the adrenal is, however, not only dependent the activity of CYP11B1, but also on the activities of upstream enzymes which include cytochrome P450 (P450) 17α-hydroxylase/17,20 lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase (3βHSD2) and P450 21-hydroxylase (CYP21A2), catalysing a network of reactions to yield substrates for CYP11B1. In addition, glucocorticoids are also produced by 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1), which catalyses the regeneration of CORT and cortisol from their respective inactive keto-metabolites, 11-dehydrocorticosterone (11-DHC) and cortisone, in peripheral tissues such as the liver and adipose tissue. Glucocorticoids regulate a wide range of physiological processes including carbohydrate, protein and lipid metabolism, immune and inflammatory processes, while also playing a key role in the stress response (Miller and Auchus 2011).

Stress is often symptomatic of an unhealthy lifestyle, and while it is also the consequence of a physical threat or the perception thereof, stress is generally defined as a state of threatened homeostasis — resulting in the activation of various physiological and behavioral adaptive compensatory responses in order to re-establish homeostasis (Pacák and Palkovits 2001; Chrousos 2009). The body responds to stress via activation of the hypothalamic-pituitary-adrenal (HPA) axis —

a complex system consisting of the hypothalamus, anterior pituitary and adrenal cortex. Activation of the HPA axis involves the release of corticotropin-releasing hormone (CRH) from the hypothalamus, which in turn stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH subsequently stimulates the adrenal cortex to release glucocorticoids - cortisol in humans and CORT in rodents. Glucocorticoids released in response to stress exert inhibitory effects on the hypothalamus and pituitary to reduce the secretion of CRH and ACTH, respectively, thereby forming a negative feedback regulatory system. During chronic stress, however, this negative feedback regulatory system is overridden, resulting in elevated basal glucocorticoid levels (Chrousos and Gold 1998; Huizenga et al. 1998). Chronically elevated glucocorticoid levels play a central role in the development of hyperglycemia, type 2 diabetes (T2D), insulin resistance, visceral obesity, dyslipidemia, atherosclerosis, hypertension and cardiovascular diseases (CVDs), which are characteristic of clinical conditions such as metabolic syndrome (MetS) and Cushing's syndrome. (Chrousos and Gold 1998; Arnaldi et al. 2003; Faggiano et al. 2003; Dekkers et al. 2007; Feelders et al. 2012).

Since Rooibos appears to have the potential to aid in the treatment of metabolic diseases associated with elevated glucocorticoid levels, the aim of this thesis was to determine the influence of Rooibos on adrenal steroid hormone production *in vitro* and *in vivo*, and in particular, on the levels of the glucocorticoids, cortisol and CORT.

In Chapter 2, Rooibos is discussed in terms of its unique flavonoid profile and the impact that processing has on flavonoid levels, however, the main focus of this chapter is the bioavailability and health-promoting properties of Rooibos.

Chapter 3 provides an overview of adrenal steroidogenesis in terms of the enzymes catalysing adrenal steroid hormone production as well as the physiological roles of these steroid hormones. While the physiological relevance of the mineralocorticoids and adrenal androgen precursors will be discussed briefly, the enzymes involved in glucocorticoid biosynthesis and the clinical conditions associated with chronically elevated glucocorticoid levels will be discussed in detail.

The aims of this thesis will be addressed in Chapter 4 and can be summarized as follows:

- to determine the influence of Rooibos and five major Rooibos flavonoids (the dihydrochalcones, aspalathin and nothofagin, their flavone analogues, orientin

and vitexin, and the flavonol, rutin) on the activities of key adrenal steroidogenic enzymes - CYP17A1, 3βHSD2, CYP21A2 and CYP11B1, expressed in COS-1 cells;

- to develop of a novel ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) method for the separation and quantification of the adrenal steroid metabolites produced by adrenal H295R cells - a human adrenal cell model expressing the steroidogenic enzymes involved in the production of the mineralocorticoids, glucocorticoids and adrenal androgens;
- to determine the influence of Rooibos and aforementioned flavonoids on overall steroid hormone production in H295R cells under basal and forskolin-stimulated conditions;
- to investigate the influence of Rooibos on the inter-conversion between cortisol and cortisone by 11βHSD1 and 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), expressed in CHO-K1 cells;
- to investigate the influence of Rooibos consumption on circulating steroid hormone levels and ratios in male Wistar rats;
- to investigate the influence of Rooibos consumption on circulating steroid hormone levels and ratios in human test subjects at risk for developing CVD.

The experimental procedures, results and discussions of this thesis are presented in the form of three published manuscripts. The first manuscript (Schloms et al. 2012) describes the influence of Rooibos and two major Rooibos flavonoids, aspalathin and nothofagin, on the activities of two key adrenal steroidogenic enzymes, CYP17A1 and CYP21A2, expressed in COS-1 cells. The influence of Rooibos, aspalathin and nothofagin was subsequently investigated on steroid hormone production in H295R cells, assayed under both basal and forskolin-stimulated conditions. Due to the complex mixture of steroid metabolites produced by the H295R cell line, a novel UPLC-MS/MS method was developed for the separation and quantification of 21 adrenal steroid metabolites. This method enabled the quantification of not only the end metabolites of adrenal steroidogenesis, but also the intermediate steroid metabolites within the mineralocorticoid-, glucocorticoid-, and adrenal androgen precursor pathways. In this manuscript, we also report, for the first time, that 11β-hydroxyandrostenedione (11OHA4), a major product of steroidogenesis in H295R cells, is a product of the hydroxylation of androstenedione (A4) by human CYP11B1.

In the second manuscript (Schloms and Swart 2014), the influence of Rooibos and the five major Rooibos flavonoids was investigated on the catalytic activities of key adrenal steroidogenic enzymes - CYP17A1, 3βHSD2, CYP21A2 and CYP11B1, expressed in COS-1 cells. The influence of the dihydrochalcones, flavones and the flavonol was subsequently investigated on steroid metabolism in H295R cells, under both basal and forskolin stimulated conditions. The structure-activity relationships of these flavonoid compounds are discussed, focusing on the structural differences of these compounds influencing their ability to inhibit individual steroidogenic enzymes and the steroid flux through the mineralocorticoid-, glucocorticoid- and adrenal androgen precursor pathways.

The third manuscript (Schloms et al. 2014) describes the influence of Rooibos consumption on circulating glucocorticoid levels and steroid ratios in male Wistar rats and human subjects at risk for CVD. In addition, the influence of Rooibos was investigated on the inter-conversion between cortisol and cortisone by 11βHSD1 and 11βHSD2 in CHO-K1 cells.

Chapter 5 presents a general discussion of the results described in Chapter 4 and highlights the main conclusions that were drawn from these findings.

CHAPTER 2

Rooibos *(Aspalathus linearis)*

2.1 Introduction

Rooibos is a popular herbal tea, or more correctly, a tisane, which is prepared from the stems and leaves of the fynbos plant, *Aspalathus linearis*, which is indigenous to the Western Cape region of South Africa. Rooibos is gaining popularity worldwide and is consumed mostly as a healthy alternative to oriental tea (*Camellia sinensis*) or coffee due to the low tannin levels, high polyphenol content and it being caffeine free. Rooibos is currently being consumed in more than 37 countries and in a recent report by the Swiss Business Hub South Africa, it was predicted to become the second most popular tea in the world after *Camellia sinensis* (Anon 2007; Joubert and de Beer 2011). Rooibos has been reported to exhibit potent anti-oxidant (Joubert et al., 2008; Yoshikawa et al., 1990), anti-inflammatory (Baba et al. 2009; Hendricks and Pool 2010; Mueller et al. 2010; Ajuwon et al. 2014), anti-carcinogenic (Petrova 2009), anti-hyperlipidemic (Beltrán-Debón et al. 2011), hypoglycemic (Joubert and de Beer 2011; Muller et al. 2012), and anti-diabetic (Mose Larsen et al. 2008; Kawano et al. 2009) properties, amongst others, suggesting a potential role for Rooibos in the overall management of metabolic diseases. In this chapter, Rooibos will be discussed in terms of the processing methods involved in the production of unfermented and fermented Rooibos tea; the unique flavonoid profile of Rooibos and how it is altered during fermentation; the bioavailability of Rooibos flavonoids and the bioactivity attributed to Rooibos extracts and some of its major flavonoid compounds.

2.2 Rooibos processing methods

Rooibos is available commercially either as a fermented or unfermented product, depending on the method by which it is processed. Traditional, fermented Rooibos is typically processed by shredding the shoots, bruising the plant material, adding water to the fermentation heap and finally, further mixing and bruising of the plant material. Shredding of the Rooibos shoots initiates enzymatic oxidation (fermentation) of the polyphenol compounds, which subsequently leads to browning of the plant material. Bruising of the plant material and the addition of water is

required to accelerate the fermentation process. Since the oxidation of the plant material requires aeration, the fermentation heap is turned over several times during the fermentation process, which lasts between 8 and 24 h, depending on the climate, composition of the plant material and processing conditions. Fermented Rooibos has a characteristic reddish-brown color and a sweet honey-like aroma.

During the production of unfermented or “green” Rooibos, oxidative changes are minimised in order to retain the green leaf color and polyphenol content, especially that of aspalathin, the principal flavonoid in unfermented Rooibos. Oxidization is minimised by inactivation of oxidative enzymes in one of the following ways: (a) by drying the plant material under vacuum; (b) by drying the shoots to a specified moisture content prior to shredding; or (c) by steaming the shoots to denature oxidative enzymes prior to shredding. Even though these techniques are very effective, there are considerable cost implications and therefore unfermented Rooibos is currently being produced by drying the shredded plant material in thin layers in the sun. This technique, however, still leads to reduced aspalathin levels and if the plant material is not correctly dried, slow browning will result in unfermented Rooibos of inferior quality. Prior to packaging, Rooibos is steam-pasteurized to ensure a low microbial content. (Joubert and Schulz 2006; Joubert et al. 2008; Joubert and de Beer 2011).

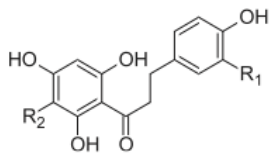
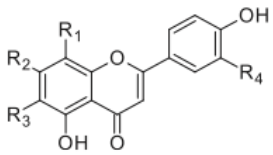
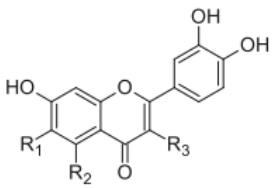
2.3 Rooibos polyphenols

Polyphenols are bioactive compounds present in a variety of dietary plant sources including fruit, vegetables and tea, amongst others. Polyphenols are generally classified as flavonoids or non-flavonoids, based on the number and structural components of their phenolic rings. Flavonoids are the largest group of polyphenols and to date, more than 9000 structurally distinct flavonoids have been identified. Based on their structural differences, flavonoids are subdivided into different classes such as the flavanols, flavanones, flavones, isoflavones, flavonols and anthocyanins (Ignat et al. 2011; Lima et al. 2014).

Rooibos contains two unique flavonoids, namely aspalathin, a dihydrochalcone *C*-glucoside (Koeppen and Roux 1965), and aspalanin, a cyclic dihydrochalcone (Shimamura et al. 2006). Another rare flavonoid present in Rooibos is the dihydrochalcone *C*-glucoside, nothofagin, which has only been identified in two

other plant species, *Nothofagus fusca* (Hillis and Inoue 1967) and *Schoepfia chinensis* (Huang et al. 2008). In addition to the dihydrochalcones, other major polyphenols identified in Rooibos include the flavones (orientin, iso-orientin, vitexin, iso-vitexin, luteolin, luteolin-7-*O*-glucoside and chrysoeriol), flavonols (quercetin, iso-quercetin, hyperoside, rutin and quercetin-3-*O*-robinobiocide), and flavanones (dihydro-orientin, dihydro-iso-orientin and hemiphlorin). Other phenolic compounds identified in Rooibos include phenolic acids, lignans, flavone diglycosides, (+)-catechin, coumarins (esculetin and esculin) and phenylpyruvic acid glucoside (PPAG) (Joubert et al. 2008; Joubert and de Beer 2011; Beelders et al. 2012). The complex polyphenol content of Rooibos is clearly illustrated in a study by Beltrán-Debón et al. (2011), in which twenty-five polyphenol compounds were identified. The structures of the major Rooibos dihydrochalcones, flavones and flavonols, as well as their relative quantities in unfermented and fermented Rooibos extracts, are shown in Table 2.1.

Table 2.1: Major flavonoids in green (unfermented) and fermented Rooibos

Structures	Compounds	Green (n=3)	Fermented (n=3)
	Dihydrochalcones		
	aspalathin (R_1=OH; R_2=*C*-glucosyl)	2.559±0.699	0.421±0.017
	nothofagin (R_1=*C*-glucosyl; R_2=OH)	0.251±0.230	0.040±0.022
	Flavones		
	orientin (R_1=*C*-glucosyl; R_2, R_4=OH; R_3=H)	0.263±0.087	0.202±0.026
	iso-orientin (R_1=H; R_2, R_4=OH; R_3=*C*-glucosyl)	0.450±0.163	0.329±0.049
	vitexin (R_1=*C*-glucosyl; R_2=OH; R_3, R_4=H)	0.042±0.020	0.035±0.009
	isovitexin (R_1, R_4=H; R_2=OH; R_3=*C*-glucosyl)	0.049±0.029	0.035±0.012
	luteolin (R_1, R_3=H; R_2, R_4=OH)	0.007±0.006	0.010±0.005
	luteolin-7-*O*-β-D-glucoside (R_1, R_3=H; R_2=*O*-glucosyl; R_4=OH)	0.015±0.008	0.015±0.008
	chrysoeriol (R_1, R_3=H; R_2=OH; R_4=OCH_3)	0.003±0.001	0.007±0.002
	Flavonols		
	quercetin (R_1=H; R_2, R_3=OH)	0.001±0.001	0.010±0.001
	hyperoside (R_1=H; R_2=OH; R_3=*O*-galactosyl)	0.021±0.012	0.016±0.015
	rutin (R_1=H; R_2=OH; R_3=*O*- rutinosyl)	0.245±0.141	0.173±0.016

Three individual plants were divided into two equal parts to prepare green (dried whole for 12h at 40°C) and fermented (shredded, fermented for 12h at 38°C and dried for 12h at 40°C) Rooibos (means ± SD; g/100g dry matter). Reproduced from Joubert and De Beer (2011) with permission from Elsevier.

Rooibos has a unique flavonoid profile that is altered during fermentation due to the oxidation of these compounds. Fermented Rooibos therefore has a lower flavonoid content, while also exhibiting less anti-oxidant activity compared to unfermented Rooibos (De Beer and Joubert 2002). The dihydrochalcones, aspalathin and nothofagin, are most affected by the fermentation process, with their levels being roughly 6-fold lower in fermented Rooibos compared to the unfermented product, as shown in Table 2.1 (Joubert and de Beer 2011). The significant reduction in the levels of aspalathin during fermentation is due to the enzymatic oxidation of this compound to its corresponding flavone analogues, iso-orientin and orientin, and it has been suggested that nothofagin would be converted to the flavones, isovitexin and vitexin, in a similar manner (Koeppen and Roux 1965; Joubert and de Beer 2011). During fermentation, aspalathin is converted to (R)/(S)-eriodictyol-6-*C*-glucoside and (R)/(S)-eriodictyol-8-*C*-glucoside. (R)/(S)-eriodictyol-6-*C*-glucoside, which is preferentially formed from aspalathin, is oxidized to iso-orientin. The conversion of iso-orientin to orientin occurs via an irreversible reaction in which the vinyl ester structure of iso-orientin opens, yielding a chalcone intermediate, which is subsequently converted to orientin (Krafczyk et al. 2009; Joubert and de Beer 2011) (Fig. 2.1). These oxidative changes could potentially account for the reduced levels of aspalathin and nothofagin following fermentation, although one would then expect that this decrease would be reflected in increased levels of their corresponding flavones. Since the levels of orientin, iso-orientin, vitexin and isovitexin remain relatively constant following fermentation, it is assumed that these compounds undergo further oxidative changes. These compounds could possibly be oxidized to the hydroxybenzoic acid derivatives 3,4-dihydroxybenzoic acid, caffeic acid, ferulic acid and vanillic acid, as these compounds have been reported to be present in higher concentrations in fermented Rooibos than in unfermented Rooibos (Richfield 2008).

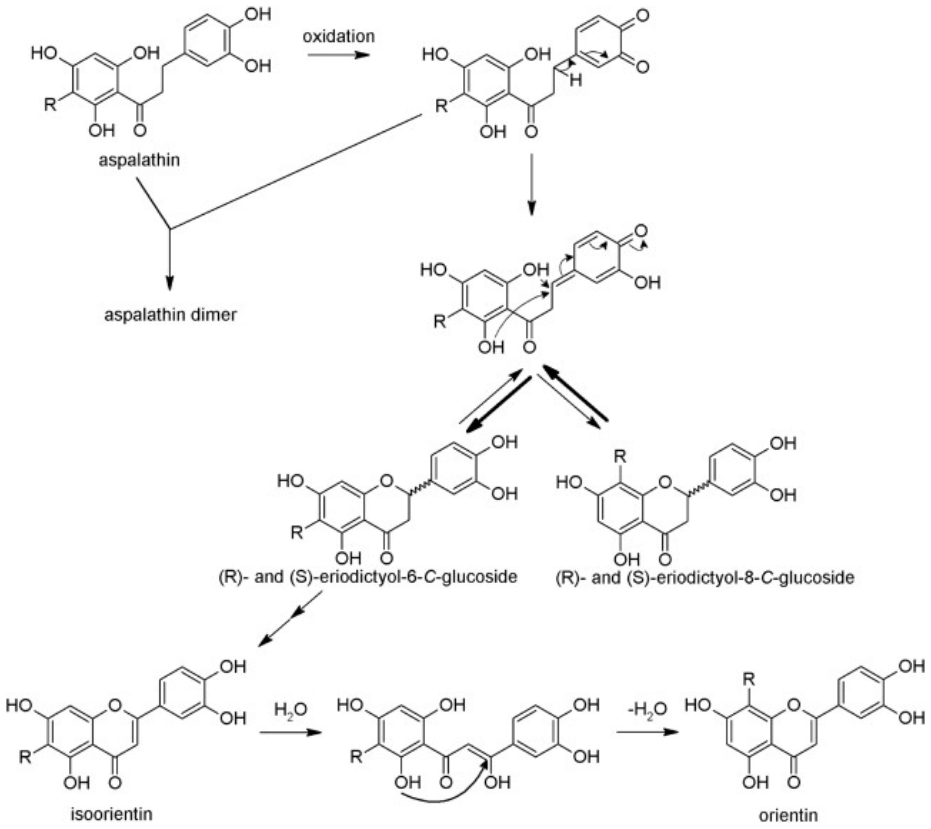


Figure 2.1: Schematic representation of aspalathin oxidation. Reproduced from Joubert and De Beer (2011) with permission from Elsevier.

In addition to the fermentation process altering the flavonoid content of Rooibos, the genetic composition of the seedling, geographical location and the particular plantation also significantly influences the flavonoid content of Rooibos (Joubert and Schulz 2006). Joubert and De Beer (2011) demonstrated considerable variations in the levels of aspalathin and orientin in unfermented Rooibos plant material obtained from 21 separate plants, which were harvested simultaneously from the same plantation. In addition, the harvest date also influenced the phenolic composition of Rooibos (Joubert and de Beer 2011). Beelders et al. (2012) subsequently developed a novel HPLC-DAD method which enables the quantification[1] of 15 of the major Rooibos polyphenols, which can be used to test the quality and authenticity of Rooibos products. The authors also clearly demonstrated

[1] Quantification of the flavonoid content of the Rooibos extracts used in the present study is shown in Addendum A.

the variation in polyphenol content between Rooibos plant material obtained from different suppliers (Beelders et al. 2012).

2.4 Bioavailability

The consumption of dietary polyphenols has been linked with a reduced risk for various diseases including T2D (Scalbert et al. 2005; Ong et al. 2011), CVD (Holt et al. 2009; Hodgson and Croft 2010), cancer (Thomasset et al. 2007; Yang et al. 2007), neurodegenerative diseases (Singh et al. 2008; Chen et al. 2009) and osteoporosis (Hagiwara et al. 2011), amongst others. In order for flavonoid compounds to exert their bioactivity, absorption in the gastrointestinal (GI) tract and transportation into circulation is crucial for these compounds to reach the target tissues. The bioavailability of a compound is the fraction of the ingested dose that is absorbed and reaches the systemic circulation. Before compounds are absorbed in the GI tract, they must become bioaccessible, which refers to the amount of ingested compound that is released from the food matrix and is available for absorption through the intestinal barrier (Velderrain-Rodríguez et al. 2014). Bioavailability can be influenced by exogenous factors such as the composition of the ingested food, the chemical formulation and dose of the ingested compound as well as the interaction and competition with co-ingested compounds. Endogenous factors influencing the bioavailability include mucosal mass, intestinal transit time, the availability of suitable receptors, metabolism, conjugation, protein binding in blood and tissues, as well as the rate of gastric emptying. Both endogenous and exogenous factors result in great inter- and intra-individual variability in bioavailability (Scholz and Williamson 2007; Holst and Williamson 2008). The intra-individual variation was clearly demonstrated in a study by Breiter et al. (2011), in which Rooibos was consumed by twelve healthy volunteers. Despite consuming equal amounts of Rooibos, the flavonoid levels detected in plasma and urine samples differed significantly between these individuals.

Following ingestion, flavonoids undergo extensive metabolism in the small intestine. Dietary flavonoids are often bound to sugar moieties in the form of beta-glycosides and deglycosylation is known to play a fundamental role in the absorption of these compounds. Within the small intestine, deglycosylation of flavonoid glycosides primarily relies on the actions of lactase-phlorizin hydrolase (LPH) and

cytosolic β-glucosidase. Following enzymatic deglycosylation, flavonoid aglycones (free flavonoids) passively diffuse across the intestinal epithelium, since the loss of the sugar moieties increases their lipophilicity (Walgren et al. 1998; Day et al. 2000; Murota et al. 2000).

Within the small intestine and liver, flavonoid aglycones are metabolized to glucuronidated-, sulfated- and methylated conjugates via the enzymatic actions of uridine-5'diphosphate glucuronosyl-transferases (UGTs), sulfotransferases (SULTs) and catechol-*O*-methyltransferases (COMTs), respectively. Conjugation of flavonoid aglycones first occurs in the small intestine, after which these metabolites are transported to the liver where they undergo further metabolism. Metabolites that reach the liver are either transported in circulation to target cells and tissues, secreted to bile and re-absorbed in the small intestine, or excreted via the urine and/or feces. The compounds that are not absorbed in the small intestine reaches the colon where colonic microflora degrades the compounds to phenolic acids, which are subsequently either excreted or re-absorbed and transported to the liver for further metabolism (Crozier et al. 2010; Thilakarathna and Rupasinghe 2013; Velderrain-Rodríguez et al. 2014). A schematic representation of human flavonoid metabolism is shown in Figure 2.2.

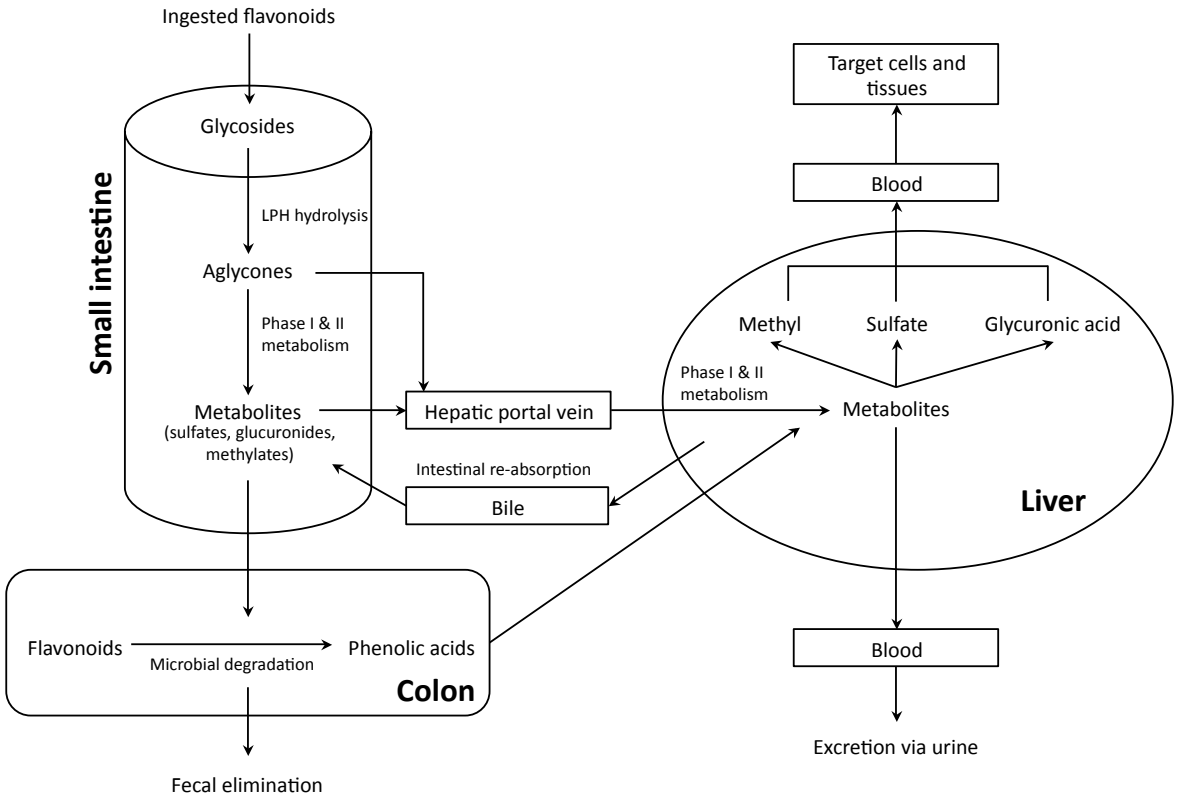


Figure 2.2. Schematic representation of flavonoid metabolism. LPH, lactase-phlorin hydrolase. Reproduced from Thilakarathna and Rupasinghe (2013).

The bioavailability of flavonoids can be assessed in one of three ways. Firstly, an increase in the total antioxidant capacity (TAC) of blood plasma after consuming polyphenol-rich foods can serve as an indirect measure of their absorption through the gut wall. This method, however, does not provide any compositional information and it has been shown that various macro- and micronutrients can also affect the TAC of plasma, either directly or through their metabolism. A more direct and accurate measurement of bioavailability is obtained via the quantification of polyphenol concentrations in plasma and urine samples following consumption of individual polyphenols or foodstuffs with known polyphenol concentrations (Scalbert and Williamson 2000; Tapiero et al. 2002; Sies 2007). Although plasma analyses are effective for the identification of circulating metabolites, it is not necessarily an accurate determination of flavonoid uptake from the GI tract. Urine analyses, on the other hand, provide a more realistic indication of GI uptake. However, these analyses do not account for metabolites absorbed at cellular level, and therefore, would not provide a true indication of absorption, and would rather reflect underestimated values (Crozier et al. 2010). A third, and the most preferred method to measure

bioavailability, includes the investigation of flavonoid uptake and distribution in tissues. The time of sampling, however, is believed to play a crucial role in the polyphenol concentration within tissues due to the kinetics involved in the absorption and elimination of polyphenols from tissues (Manach et al. 2004).

The first *in vivo* study to investigate the bioavailability of Rooibos was performed by Kreuz et al. (2008). In this study, aspalathin metabolism was assayed in pigs following oral administration of an unfermented Rooibos extract over a period of 11 days. Six metabolites were identified in the urine which included: intact aspalathin, methylated aspalathin, glucuronidated aspalathin, methylated- and glucuronidated aspalathin, a glucuronidated aglycone of aspalathin and a metabolite of eriodictyol. The data obtained from this study indicated that aspalathin is either absorbed in the small intestine as a *C*-glycoside, or cleaved into an aglycone. The major metabolite detected in the urine was methylated aspalathin. Only 0.1% to 0.9% of the administered dose of aspalathin was detected in urinary samples, while no metabolites were detected in the plasma (Kreuz et al. 2008).

In a subsequent study by Courts and Williamson (2009), the bioavailability of Rooibos was investigated in human subjects following the consumption of an unfermented Rooibos extract. Glucuronidated- and methylated metabolites of aspalathin were observed in the urine following Rooibos ingestion, confirming that aspalathin is absorbed, methylated and glucuronidated *in vivo*. In addition, methylated metabolites of intact (glycosylated) aspalathin were identified in urine samples, suggesting that *C*-glycosyl flavonoids does not have to be deglycosylated in order to be absorbed in humans (Courts and Williamson 2009). These data corroborated previous findings by Kreuz et al. (2008).

In the same year, Stalmach et al. (2009) detected eight metabolites of aspalathin in the urinary samples of humans following a single intake (500 ml) of either an unfermented or fermented Rooibos tea beverage. These metabolites included *O*-linked methylated-, sulfated- and glucuronidated metabolites of aspalathin and eriodictyol-*O*-sulfate. The main compound excreted following the consumption of the unfermented and fermented drink was *O*-methyl-aspalathin-*O*-glucuronide and eriodictyol-*O*-sulfate, respectively. The majority of aspalathin metabolites were detected within the first 5 h following intake, which is suggestive of small intestine absorption. However, further analyses of the samples showed that aspalathin metabolites and eriodictyol-*O*-sulfate comprised only 0.22% of the 159 µmol total

polyphenols consumed in the unfermented Rooibos beverage, while these metabolites comprised 0.09% of the 84 µmol total polyphenols consumed in the fermented Rooibos beverage. The unfermented and fermented Rooibos beverage contained 90 µmol and 8 µmol aspalathin, respectively, with only 317 nmol and 14 nmol aspalathin metabolites being excreted over a 24 h period. No flavonoid compounds were detected in the plasma (Stalmach et al. 2009).

Even though the levels of aspalathin ingested by pigs during the study conducted by Kreuz et al. (2008) was roughly 400 times higher than the single dose administered in the study performed by Stalmach et al. (2009), the urinary recovery rates of aspalathin metabolites in these two studies would seem to suggest that limited aspalathin absorption occurred, regardless of the administered dose. However, taking into account the significantly low recovery rates of aspalathin obtained in both studies, it is possible that these low levels could be attributed to inadequate extraction procedures. In the studies conducted by both Kreuz et al. (2008) and Stalmach et al. (2009), polyphenol compounds bound to serum proteins were not considered, and protein-polyphenol complexes had not been denatured prior to extraction.

These protein-polyphenol complexes were, however, taken into account in a subsequent human study by Breiter et al. (2011), investigating the bioavailability of flavonoids following the consumption unfermented Rooibos tea. In contrast to the previous studies, this was the first group to detect intact flavonoids in the plasma of humans following Rooibos consumption. The compounds identified in plasma samples included aspalathin, orientin, iso-orientin, vitexin, (S)-eriodictyol-8-*C*-glucoside, eriodictyol-7-*O*-glucoside and quercetin-*O*-rutinoside. The recovery rates of these flavonoids were, however, also very low, ranging between 0.2% and 2.3%. Intact aspalathin and nothofagin as well as seven of their metabolites were identified in urinary samples. These metabolites included sulfated-, glucuronidated-, methylated-, both methylated- and glucuronidated metabolites, as well as the aglycone metabolites of aspalathin and nothofagin (Breiter et al. 2011).

2.5 Biological activity

Even though Rooibos flavonoids have been reported to have poor bioavailability *in vivo*, consumption of water extracts of Rooibos has been linked to anti-oxidant, anti-inflammatory, anti-tumor, anti-carcinogenic, hepatoprotective, phyto-estrogenic, immune-modulatory, anti-hyperlipidemic and hypoglycemic effects, amongst others (Joubert et al. 2008; Joubert and de Beer 2011). Studies reporting on the biological properties of Rooibos have been extensively reviewed by Joubert et al. (2008) and Joubert & De Beer (2011). The main focus of this section will be on the health promoting properties of Rooibos associated with reduced risks for metabolic related diseases such as diabetes and CVDs.

2.5.1 Anti-oxidative properties of Rooibos

Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are implicated in cellular signaling systems, receptor activation and gene expression, and can have both beneficial and harmful biological properties. Oxidative damage, also termed ‘oxidative stress’ occurs when excessive free radical production is not prevented by endogenous anti-oxidant mechanisms. Elevated levels of free radicals cause damage to nucleic acids, membrane lipids, as well as structural and functional proteins. Oxidative stress can therefore be defined as an imbalance between pro-oxidant and anti-oxidant levels in the body, with elevated pro-oxidant levels leading to oxidative damage (Halliwell 2006, 2007; Darvesh et al. 2010). Various studies have confirmed that oxidative stress resulting from excessive free radical production plays a crucial role in the development of age-related diseases such as neurodegenerative diseases, CVDs, diabetes and cancer (Rao and Balachandran 2002; Emerit et al. 2004; Halliwell 2006; Valko et al. 2006; Wang et al. 2006).

Polyphenols are known to exhibit potent anti-oxidant properties and the use of these compounds in the treatment of several chronic diseases has proved to be successful (Linseman 2009). Glutathione (GSH) is the most potent intracellular antioxidant, and the ratio between reduced GSH and oxidized GSH (GSH:GSSG) is used as a marker to determine the anti-oxidative capacity of the cell, with low GSH levels and GSH:GSSH ratios being indicative of oxidative stress (Exner et al. 2000;

Franco et al. 2007). GSH has been reported to counteract ageing, various cancers and heart diseases (Townsend et al. 2003; Exner et al. 2000; Locigno and Castronovo 2001; Franco et al. 2007; Ballatori et al. 2009). It has, however, been shown that GSH levels decrease with age (Marí et al. 2009) and due to smoking (Teramoto et al. 1996). In addition, GSH cannot be taken as a supplement since it degrades in the gut (Witschi et al. 1992).

In a study by Marnewick et al. (2003), it was shown that rats consuming Rooibos tea as their only source of liquid for 10 weeks had a 5-fold increase in the GSH:GSSG ratio (Marnewick et al. 2003). These results were corroborated in a more recent study by Ajuwon et al. (2014), investigating the effect of Rooibos on lipopolysaccharide (LPS)-induced oxidative stress in Wistar rats. Rooibos consumption was shown to significantly suppress LPS-induced oxidative stress and inflammatory responses in the livers of rats by reducing liver damage, lipid-peroxidation, the secretion of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and by preventing the LPS-induced reduction of GSH:GSSH ratios (Ajuwon et al. 2014). In a study by Hong et al. (2014), the protective action of Rooibos was clearly demonstrated in rats subjected to chronic immobilization stress. Rooibos was shown to regulate GSH metabolism by counteracting the reduction in GSH levels and GSH:GSSG ratios caused by immobilization-induced oxidative stress. In addition, Rooibos reversed the increase in stress related metabolites, prevented lipid-peroxidation and stress-induced protein degradation, while also modulating changes in anti-oxidative enzyme activities (Hong et al. 2014).

The impact of dietary choices on oxidative stress is underscored in the study by Francisco (2010), in which the consumption of sucrose with a meal containing oxidized and/or oxidizable lipids was shown to increase oxidative stress, also referred to as post-pranadial oxidative stress. In this study, a single oral dose of Rooibos containing sucrose, together with a high fat meal, inhibited post-pranadial oxidative stress in healthy humans. Rooibos reduced lipid-oxidation biomarkers, increased the anti-oxidant capacity and restored the redox status as reflected in the increased plasma GSH levels (Francisco 2010). These findings were supported in a subsequent study by Marnewick et al. (2011), reporting that the consumption of six cups of Rooibos tea per day over a period of six weeks reduced oxidative stress by

decreasing lipid-peroxidation and increasing the GSH:GSSG ratio in humans at risk for CVD (Marnewick et al. 2011).

2.5.2 Anti-diabetic properties of Rooibos

Diabetes mellitus is a metabolic disorder characterized by chronically elevated glucose levels (hyperglycemia) – either due to a deficiency in insulin secretion and/or the body's resistance to insulin, a hormone that regulates glucose utilization. Type 1 diabetes (T1D) is associated with insulin deficiency as a result of autoimmune-mediated deletion of pancreatic β-cells, while T2D is characterized by the inability of tissues to respond to insulin (insulin resistance), even if insulin levels are normal. Pancreatic β-cells mediate glucose uptake via the glucose transporter, GLUT2, when blood glucose levels rise, which ultimately leads to insulin secretion (Braun et al. 2008; Leto and Saltiel 2012). The secretion of insulin results in a number of regulatory processes: GLUT4-dependent glucose uptake in various tissues including skeletal muscle and adipose tissue is increased, glycogenesis in the liver is stimulated, while lipogenesis is promoted and lipolysis is inhibited in adipose tissue. As circulating glucose levels start to decline, pancreatic α-cells are stimulated to release glucagon, which promotes gluconeogenesis and glycogenolysis in the liver and lipolysis in adipose tissue. In patients with T2D, defects in the regulation of these hormones lead to sustained elevated blood glucose levels, which have a wide range of pathological consequences. Insulin is used for the treatment of T1D, while drugs that target glucose-regulating processes in the pancreas, gut, skeletal muscle, adipose tissue or liver is used for the treatment of T2D (Mohler et al. 2009).

Natural plant products are often included in the treatment of non-insulin dependent diabetes, particularly within traditional medicines in developing countries. The first study investigating the anti-diabetic properties of fermented Rooibos found that diabetic parameters tested in streptozotocin[2] (STZ) - induced diabetic rats were unaffected by Rooibos extracts. Rooibos was, however, suggested to aid diabetic complications such as ocular pathological changes (Uličná et al. 2006). In a subsequent study by Kawano et al. (2009), it was shown that aspalathin, the major flavonoid compound in unfermented Rooibos, exhibited hypoglycemic effects. *In*

[2] STZ is used to reduce/deplete insulin producing cell numbers and induce hyperglycemia to levels typically found during T1D or late stage T2D.

vitro, aspalathin significantly and dose-dependently increased the uptake of glucose by L6 myotubes (rat skeletal muscle-derived cell line) while also significantly increasing insulin secretion from RIN-5F cells (rat-derived pancreatic β-cell line). *In vivo*, aspalathin ameliorated impaired glucose tolerance in T2D model *db/db* mice, while also reducing increased fasting plasma glucose levels (Kawano et al. 2009). These results were corroborated in a more recent study by Son et al. (2013), investigating the mechanisms of action to which the anti-diabetic effects of aspalathin could be attributed. Aspalathin dose-dependently increased glucose uptake by L6 myotubes through GLUT4 translocation to the plasma membrane, via the activation of AMP-activated protein kinase (AMPK)**.** In RIN-5F cells, aspalathin reduced the advanced glycation end product-induced increase in ROS due to its radical scavenging capabilities. *In vivo*, aspalathin improved hyperglycemia and impaired glucose intolerance in diabetic *ob/ob* mice, while also reducing hypertriglyceridemia. The expression of genes encoding enzymes catalyzing gluconeogenesis, glycogenolysis and lipogenesis were suppressed by aspalathin. In addition, aspalathin reversed the reduced expression of genes encoding enzymes catalysing glycogenesis in the liver, thus possibly contributing towards reducing blood glucose and serum triglyceride (TG) levels (Son et al. 2013).

In an earlier study by Muller et al. (2012), the hypoglycemic potential of an unfermented Rooibos extract, as well as that of aspalathin and rutin was investigated (Muller et al. 2012). Rutin (quercetin-3-*O*-rutinoside) levels are significantly higher in unfermented Rooibos than in fermented Rooibos extracts and it has been shown to inhibit β-glucosidase activity (Li et al. 2009), increase plasma insulin levels and reduce plasma glucose levels in STZ-induced diabetic rats (Kamalakkannan and Prince 2006). In their study, Muller et al. (2012) showed glucose uptake to be stimulated in C2C12 cells (murine myoblast cell model) in a dose-dependent manner by the unfermented Rooibos extract, while stimulation of glucose uptake was not detected at lower concentrations in the Chang liver (HeLa derivative) cell line expressing negligible GLUT4. In C2C12 cells, aspalathin and rutin did not result in dose-dependent increases in glucose uptake, with rutin stimulating glucose uptake only at 100 μM, while aspalathin stimulated glucose uptake significantly from 1 – 100 μM. The increases in glucose uptake detected in Chang cells were not shown to be significant. In STZ-induced diabetic rats, the Rooibos extract reduced glucose levels to a similar extent as in the presence of metformin, while a glucose tolerance test

indicated that Rooibos was significantly better at lowering circulating glucose levels than vildagliptin, a dipeptidyl peptidase-4 inhibitor. Analyses of the synergistic combined effects of an equimolar aspalathin:rutin mixture showed that maximal glucose lowering effects were obtained 4 h after administration, with a ± 3-fold greater reduction compared to single administrations of aspalathin and rutin. After 4 h, the extract exhibited similar glucose lowering effects at concentrations ranging between 5 - 50 mg/kg body weight. It is interesting to note that neither compound was capable of stimulating glucose uptake *in vitro* or *in vivo* as significantly as the Rooibos extract, even though aspalathin and rutin levels were significantly lower in the extract. The highest stimulation of glucose uptake in C2C12 cells was achieved at 5 µg extract/mL, which contained 2.2 µM aspalathin and 0.04 µM rutin (Muller et al. 2012). These data indicate the presence of more potent compounds or synergistic interactions within the complex mixture of Rooibos polyphenols.

Following various *in vivo* studies reporting on the hypoglycemic properties of fermented Rooibos extracts as well as the Rooibos flavonoids, aspalathin and rutin, Rooibos was patented as an anti-diabetic agent in 2011. The patent[3] claims the use of Rooibos extract, aspalathin and rutin in the prevention and treatment of diabetes, based on the ability of the extract and flavonoid compounds to improve impaired glucose tolerance and to reduce plasma glucose levels in STZ-induced diabetic rats and diet-induced T2D monkeys. Interestingly, when aspalathin and rutin were administered to diabetic rats, similar results were obtained as those in the presence of the Rooibos extract. It was also reported that aspalathin and rutin were most effective when used in combination. Aspalathin alone, or in particular when used together with rutin, significantly lowered plasma glucose levels, while also generally alleviating diabetic symptoms in both type 1- and type 2 diabetic rats. As anti-diabetic agents, a human dosage of aspalathin, or aspalathin in combination with rutin, was suggested to be administered between 0.1 – 50 mg/kg/day, while the dosage of fermented Rooibos extract should range between 1 – 2.5 mg/kg/day (Mose Larsen et al. 2008).

In addition to aspalathin, rutin and Rooibos extracts exhibiting hypoglycemic properties, Mathijs et al. (2014) recently showed that phenylpropenoic acid glucoside

[3] European Patent No. EP 2 120 980 B1 (WO 2008/110551).

(PPAG), a polyphenol compound isolated from Rooibos, also exhibited hypoglycemic properties in mice consuming a high-fat, fructose-containing diet to induce obesity and hyperglycemia. Treatment with PPAG prevented diet-induced hyperglycemia in these mice, while also leading to a 3-fold increase of β-cell mass. In addition, PPAG protected pancreatic β-cells against apoptosis (Mathijs et al. 2014). Rooibos flavonoids including quercetin, rutin and luteolin, have also been shown to protect pancreatic β-cells from experimental agents that induce diabetes. In a study by Coskun et al. (2005), it was shown that quercetin prevented and protected rat pancreas against β-cell damage and STZ-induced oxidative stress (Coskun et al. 2005). In a similar way, rutin and apigenin (aglycone of vitexin) also prevented STZ-induced oxidative stress and increased insulin release in rat pancreas islets (Esmaeili et al. 2009). Furthermore, it was shown that apigenin, quercetin and luteolin inhibited cytokine-induced pancreatic β-cell damage *in vitro* (Kim et al. 2007). The enhancement of insulin secretion from pancreatic β-cells is a standard approach for the treatment of diabetes and various studies have reported that quercetin and rutin both increase insulin secretion (Hii and Howell 1985; Pinent et al. 2008; Yang et al. 2010).

Numerous studies have indicated a link between diabetes and oxidative stress (Kesavulu et al. 2000; Tinahones et al. 2009; Hoehn et al. 2010). As the severity of diabetes progresses, the formation of ROS increases and it is now widely accepted that oxidative stress plays a prominent role in the development of diabetes and associated complications (Schleicher and Weigert 2000; Brownlee 2005; Houstis et al. 2006). Adipose tissue includes a variety of cell types such as adipocytes, preadipocytes, endothelial cells and immune cells, which consist of lymphocytes and bone marrow-derived macrophages. The number of macrophages present in adipose tissue has been shown to correlate with the degree of obesity, as the levels of macrophages are significantly higher in obese individuals compared to lean individuals (Weisberg et al. 2003). Macrophages that accumulate in the adipose tissue are a major source of the pro-inflammatory cytokines, TNF-α and IL-6. TNF-α levels are elevated in the blood and adipose tissue of obese animals, and neutralization of TNF-α has been shown to improve insulin sensitivity (Hotamisligil 1999; Shoelson et al. 2006). Furthermore, TNF-α and IL-6 released by adipocytes and macrophages were shown to be elevated in diabetic and insulin resistant patients (Senn et al. 2002). Consistent with the findings showing that inflammatory

markers are upregulated in individuals with diabetes, insulin has also been shown to downregulate free fatty acid (FFA) release and numerous inflammatory markers (Kim et al. 2006; Dandona et al. 2009). The crosstalk between inflammation and ROS in diabetes is also apparent, since ROS has been shown to promote macrophage infiltration, adipocyte senescence as well as the production of TNF-α and IL-6 (Rahman et al. 2002). It is therefore likely that agents capable of suppressing inflammation by reducing pro-inflammatory cytokine levels and/or their biological actions may have beneficial effects in the treatment of insulin resistance and/or diabetes (Kim et al. 2004; Larsen et al. 2007; Sears and Ricordi 2012; Gómez-Zorita et al. 2013; Siriwardhana et al. 2013). Besides the antioxidant and hypoglycemic properties attributed to Rooibos, it has also been reported to exhibit anti-inflammatory effects both *in vitro* (Hendricks and Pool 2010; Mueller et al. 2010) and *in vivo* (Baba et al. 2009). Preliminary results from studies in our group have shown that Rooibos decreased IL-6 production in rat adrenal tissue, while significantly increasing the levels of interleukin-10 (IL-10), an anti-inflammatory cytokine ($P<0.05$) (unpublished data). It is therefore possible that the anti-diabetic properties of Rooibos could, in part, also be ascribed to its anti-inflammatory properties.

2.5.3 Cardio-protective properties of Rooibos

CVDs are the leading cause of death in the world and the main risk factors contributing to CVDs include an unhealthy diet, a lack of physical activity, smoking, obesity, hypertension, diabetes and elevated lipid levels[4]. Although Rooibos has been anecdotally reported to aid hypertension, studies investigating the cardio-protective properties of Rooibos are limited. The first scientific study investigating the effect of Rooibos on angiotensin-converting enzyme (ACE) - a crucial enzyme involved in the rennin-angiotensin aldosterone system (RAAS), and commonly targeted in the treatment of hypertension and CVD, was carried out by Persson et al. (2006). In comparison to green tea (Japanese Sencha) and black tea (Indian Assam Broken Orange Pekoe), Rooibos did not inhibit ACE significantly at the

[4] WHO, 2014 - Cardiovascular diseases: Fact sheet No.317.

http://www.who.int/mediacentre/factsheets/fs317/en/ (Updated March 2013).

concentrations assayed in HUVEC cells (cultured human umbilical endothelial cells). However, nitric oxide production was significantly lower in these cells when exposed to Rooibos compared to green and black tea (Persson et al. 2006). In a subsequent *in vivo* study investigating the inhibition of ACE by green tea, black tea and Rooibos in human subjects, a single oral dose of Rooibos or green tea significantly inhibited the activity of ACE, suggesting that both teas may have cardio-protective properties. No effect was, however, observed on blood pressure after tea consumption (Persson et al. 2010). The authors subsequently showed mixed inhibition of ACE by both Rooibos and green tea, however, neither were as efficient as enalaprilat (Persson 2012).

As previously discussed, the investigation by Francisco (2010) showed that a single oral dose of Rooibos extract containing sucrose, together with a high fat meal, decreased the levels of plasma insulin, glucose, total cholesterol, low density lipid (LDL) cholesterol, TGs and inflammatory biomarkers (Francisco 2010). These findings were confirmed in a subsequent human study by Marnewick et al. (2011), reporting that Rooibos consumption significantly improved the lipid profiles by reducing LDL cholesterol and TG levels, while increasing the levels of high density lipid (HDL) cholesterol in humans at risk for CVD (Marnewick et al. 2011). In a more recent study by Dludla et al. (2014) investigating diabetic cardiomyopathy (DCM), a disorder of the heart muscle associated with diabetes, Rooibos was shown to have cardio-protective effects in primary cardiomyocytes cultures isolated from diabetic rats. Rooibos reduced intracellular ROS and cell death under experimentally induced oxidative-stress and ischemic conditions. In addition, Rooibos maintained normal intracellular ATP and GSH levels, which if depleted, are strongly associated with oxidative stress and metabolic dysfunction. It was suggested that by maintaining the balance between ROS and antioxidants, Rooibos was able to protect cardiomyocytes from cell damage associated with disease states such as diabetes (Dludla.2014).

Going hand in hand with T2D, hypertension and CVD, is obesity, a chronic disease commonly associated with poor lifestyle. The beneficial properties of Rooibos regarding metabolic conditions such as hyperglycemia, hyperlipidemia, and CVDs, as discussed above, is suggestive of Rooibos exhibiting anti-obesity potential. However, only two studies have been reported to date. In the first study by Beltrán-Debón et al. (2011), the effect of Rooibos on dietary-induced hyperlipidemia in male LDLr−/− mice, a mouse model representing aspects resembling MetS, was

investigated. Continuous Rooibos consumption resulted in significant reductions in the levels of serum cholesterol, TGs and FFAs in rats consuming a high fat diet. In addition, the effects of Rooibos were more prominent than in the chow fed rats and resulted in significantly improved VLDL:LDL:HDL ratios. Analysis of adipose tissue showed that Rooibos promoted lipid accumulation in adipocytes of rats consuming standard rat chow, however, in rats consuming a high fat diet, Rooibos protected the liver from lipid storage without the accumulation of fat in adipocytes. Analyses of liver tissue showed that Rooibos prevented dietary-induced hepatic steatosis in hyperlipidemic mice, which was also associated with a significant decrease in macrophage recruitment, a reduction not detected in the rats fed a normal diet. In the 3T3-L1 pre-adipocyte cell line, Rooibos was shown to decrease TG levels in a dose-dependent manner in differentiated cells, while not affecting differentiation of pre-adipocytes (Beltrán-Debón et al. 2011). In a subsequent study, a fermented Rooibos extract was shown to modulate adipocyte differentiation in 3T3-L1 cells. Rooibos exhibited anti-adipogenic effects in differentiating 3T3-L1 preadipocytes, while also reducing lipid accumulation in these cells, possibly by inhibiting the expression of enzymes involved in adipogenesis and lipid metabolism. In addition, glucose uptake and ATP levels were increased in differentiating 3T3-L1 adipocytes. Interestingly, leptin levels in 3T3-L1 adipocytes were reduced in the presence of Rooibos (Sanderson et al. 2014).

2.5.4 Modulation of adrenal steroidogenic enzymes by Rooibos

Adrenal steroid hormones, which include the mineralocorticoids, glucocorticoids and adrenal androgens, are responsible for the regulation of a variety of physiological processes in the body. These steroid hormones are produced in the adrenal gland by P450 enzymes and hydroxysteroid dehydrogenases (HSDs). Abnormal adrenal steroid hormone levels due to altered enzyme activities impact a number of clinical conditions such as hypertension, MetS, T2D and heart failure, to name but a few, and will be discussed in greater detail in Chapter 3.

P450 enzymes and flavonoids have been reported to interact with each other in at least three ways − flavonoids have been shown to induce the biosynthesis of P450 enzymes; alter (inhibit / stimulate) the enzymatic activities of P450 enzymes, while also serving as substrates for several P450 enzymes. Besides the effect on the

catalytic activity of steroidogenic enzymes, flavonoids have also been shown to influence physiological processes by interfering at the receptor level. In addition, flavonoids are often referred to as phytoestrogens due to the structural similarity to estrogen and the ability to bind to the estrogen receptor (ER). Various flavonoids have been reported to inhibit the activity of cytochrome P450 aromatase (CYP19), a key enzyme involved in estrogen biosynthesis and a target for the treatment of breast cancer (Hodek et al. 2002). Luteolin, a flavonoid compound present in Rooibos, has for example, been shown to inhibit estrogen biosynthesis by reducing the expression of CYP19 (Lu et al. 2012).

The influence of flavonoids on adrenal steroidogenesis has been investigated by several groups. Previous studies in our laboratory by Richfield (2008) and Perold (2009) showed that Rooibos extracts significantly inhibited the binding of substrates, progesterone (PROG) and 17OH-progesterone (17OH-PROG), to enzymes in ovine adrenal microsomal preparations. These preparations contain both CYP17A1 and CYP21A2, which catalyze the conversion of pregnenolone (PREG) and PROG in adrenal steroidogenesis. Subsequent substrate conversion assays in COS-1 cells showed that Rooibos significantly reduced PROG conversion by CYP17A1 and CYP21A2, with CYP21A2 being inhibited to a greater extent than CYP17A1. In addition, it was also shown that the inhibitory effect of an unfermented Rooibos extract was greater than that of a fermented Rooibos extract (Richfield 2008; Perold 2009).

Although assays in COS-1 cells yield data regarding the interaction between flavonoids and individual steroidogenic enzymes, studies conducted in H295R cells, an adrenal carcinoma cell line expressing all of the adrenal steroidogenic enzymes, provide a broader perspective as these analyses are indicative of the effect of flavonoids on overall adrenal steroid production. In a study by Mesiano et al. (1999), it was shown that both genistein and daidzein inhibit cortisol production in cAMP-stimulated H295R cells as well as in ACTH-stimulated cultured fetal and postnatal adrenal cortical cells. The authors proposed that the reduction observed in cortisol levels was due to inhibition of CYP21A2, since both genistein and diadzein inhibited cortisol production when PROG and 17OH-PROG were added to the medium. However, when deoxycortisol was added, no inhibition on cortisol production was detected, indicating that the compounds do not inhibit CYP11B1 (Mesiano et al. 1999). In a subsequent study by Ohno et al. (2002), the flavonoids, daidzein,

genistein and 6-hydroxyflavone was shown to selectively inhibit the activities of CYP17A1, 3βHSD2, CYP21A2 and CYP11B1 in H295R cells. In addition, these flavonoids also significantly inhibited cortisol production in cAMP stimulated H295R cells (Ohno et al. 2002). The authors subsequently showed that genistein significantly reduced serum CORT and testosterone levels in rats (Ohno et al. 2003). In a more recent study, Ohlsson et al. (2010) showed that daidzein and genistein inhibited cortisol and testosterone production in H295R cells, while an equimolar mixture of diadzein, genistein and vitexin inhibited the production of cortisol, testosterone and aldosterone (ALDO) in an additive manner (Ohlsson et al. 2010).

Although the aforementioned Rooibos flavonoids, luteolin and vitexin, was shown to inhibit CYP19 as well as the production of end products in H295R cells, respectively, the former compound was identified in fermented Rooibos only and is present at very low levels. Vitexin, on the other hand, appears to be relatively stable and is present at similar levels in both fermented and unfermented Rooibos (Richfield 2008). In our studies, we have shown that vitexin's interaction with key adrenal steroidogenic enzymes is complex in terms of substrate preference. The interaction of Rooibos extracts and the major flavonoids in Rooibos with adrenal steroidogenic enzymes *in vitro* and *in vivo* will be discussed in Chapter 4 and is presented as three published manuscripts. The relevant literature regarding the interaction between flavonoid compounds and steroidogenic enzymes will also be addressed.

2.6 Summary

From the data presented in this chapter it is evident that Rooibos exhibits various health promoting properties that impact metabolic disorders. It is also clear that oxidative stress plays a critical role in the etiology of metabolic diseases. Changes in the redox status that occur during oxidative stress as a result of excessive free radical production can be counteracted by polyphenols exhibiting potent anti-oxidant properties. The anti-oxidant properties of Rooibos have been widely reported and its ability to restore the redox imbalance is reflected in positive changes in GSH:GSSH ratios in rats and humans. It is thus possible that decreased GSH:GSSH ratios may impact on diabetes, since the link between oxidative stress and diabetes has been clearly established. Rooibos flavonoids are able to protect pancreatic β-cells against oxidative stress and cell damage, while also exhibiting

hypoglycemic effects *in vitro* and *in vivo*, as reflected in increased glucose uptake, improved glucose tolerance and the stimulation of insulin secretion. The protective role of Rooibos is also evident in heart muscle disorders associated with diabetes, with Rooibos protecting cardiomyocytes by maintaining the balance between ROS and antioxidants. The cardio-protective effects of Rooibos are demonstrated both *in vitro* and *in vivo*, with *in vivo* studies reporting reductions in plasma levels of total cholesterol, LDL cholesterol and TGs, while the levels of HDL cholesterol is increased following Rooibos consumption. Although the anti-obesity properties of Rooibos appear to be less apparent, extracts were able to modulate adipocyte differentiation, enzyme expression and lipid metabolism. However, the anti-diabetic and cardio-protective properties of Rooibos as discussed above are all factors that would also impact on obesity parameters.

The properties of Rooibos discussed in this chapter therefore suggest a therapeutic role for Rooibos in the overall management of endocrine-related disorders. In addition, Rooibos extract, as well as specific Rooibos flavonoids have been shown to alter the activities of key enzymes in adrenal steroidogenesis, which also plays a fundamental role in the regulation of the endocrine system. It therefore seems plausible that Rooibos extract and/or Rooibos flavonoid compounds could modulate adrenal steroidogenic enzyme activities and reduce the levels of the glucocorticoids, which would further add to the anti-diabetic and cardio-protective properties of Rooibos. Since the aim of the present study is to investigate the influence of Rooibos on adrenal steroidogenesis, the next chapter will provide an overview of adrenal steroidogenesis in terms of the enzymes involved in adrenal steroid production, as well as the physiological roles of these hormones in normal and diseased states.

CHAPTER 3

Adrenal steroidogenesis

3.1 Introduction

Adrenal steroid hormones regulate a variety of essential developmental and physiological processes in the body. These hormones are synthesised in the adrenal cortex from the common precursor, cholesterol, through a network of enzyme-catalysed reactions within the adrenal steroidogenic pathway. Based on their physiological functions, adrenal steroid hormones can be divided into three classes, namely the glucocorticoids, mineralocorticoids and adrenal androgen precursors. The adrenal androgen precursors are involved in sexual growth and development, while the mineralocorticoids regulate sodium and water homeostasis. Glucocorticoids play a key role in the regulation of carbohydrate-, protein- and lipid metabolism, immune and inflammatory responses and the stress response. Abnormal steroid hormone levels, due to altered enzyme activities and/or expression levels, have been implicated in numerous clinical conditions. Elevated glucocorticoid levels due to chronic stress, for instance, have been linked with the development of visceral obesity, hypertension, T2D and CVDs, amongst others. In this chapter, adrenal steroidogenesis will be discussed in terms of the enzymes catalyzing adrenal steroid hormone production, as well as the physiological effects of the mineralocorticoids, glucocorticoids and adrenal androgens and their role in disease. The main focus, however, will be on the glucocorticoids and clinical conditions that are associated with stress-induced elevation of glucocorticoids. In order to understand how stress can cause disease, it is necessary to first understand the mechanisms by which adrenal steroid hormones are produced and regulated.

3.2 Anatomy and morphology of the adrenal gland

The adrenals are small endocrine glands situated on the upper pole of each kidney. The mammalian adrenal gland consists of two structurally and functionally distinct types of endocrine tissue, namely the outer adrenal cortex and the inner adrenal medulla (Fig. 3.1). The adrenal medulla consists of catecholamine producing

chromaffin cells, while the adrenal cortex contain steroid producing cortical cells. This is, however, rather an oversimplification, since, chromaffin cells are also found in the cortex, either in the form of islets or extending from the medulla through the cortex, while cortical cells are also present in the medulla as islets surrounded by chromaffin cells (Palacios and Lafarga 1975; Bornstein et al. 1991; Bornstein and Ehrhart-Bornstein 1992). The medullary chromaffin cells, which originate from neural crest material, mainly secrete the catecholamines, epinephrine (adrenaline) and norepinephrine (noradrenaline), along with various other neurotransmitters and neuropeptides (Winkler et al. 1986). Since these two endocrine tissues are morphologically interwoven, their functions have also been shown to interact with each other – chromaffin cells are able to regulate steroid release from the adrenal cortex, while adrenal steroids can induce the production of catecholamines in the medulla (Ehrhart-Bornstein and Bornstein 2008).

The adrenal cortex consists of three distinct adrenal zones namely the *zona glomerulosa*, *zona fasciculata* and *zona reticularis,* which differ from each other with regards to their morphological features as well as the steroid hormones that they secrete (Fig. 3.1) (Ehrhart-Bornstein et al. 1998).

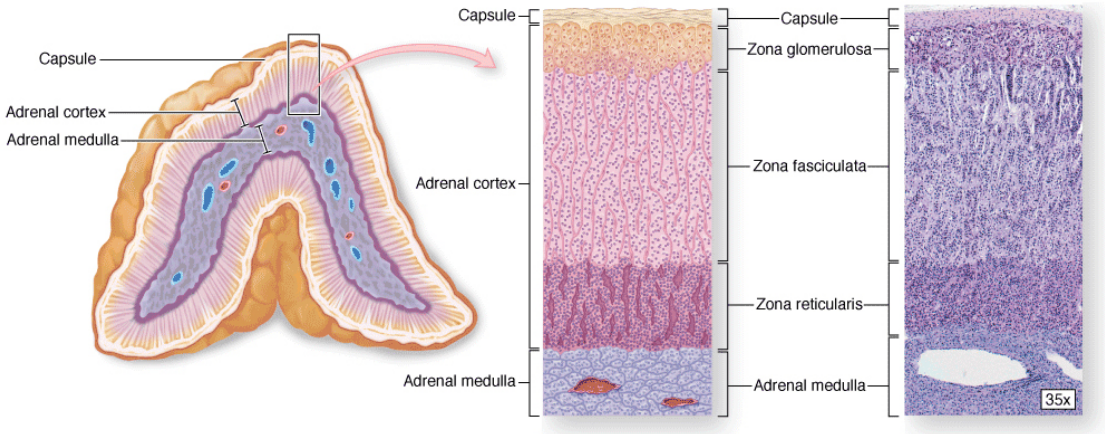


Figure 3.1: Schematic cross-section of the adrenal gland showing the capsule, adrenal cortex and medulla. The adrenal cortex consists of the *zona glomerulosa, zona fasciculata* and *zona reticularis,* which produce the mineralocorticoids, glucocorticoids and adrenal androgen precursors, respectively. Reproduced from Mescher et al. (2010) with permission from McGraw Hill.

The outer *zona glomerulosa* cells, which comprise ± 15% of the adrenal cortex volume, secrete the mineralocorticoid, ALDO. The *zona glomerulosa* is situated directly beneath the fibrous connective tissue capsule and the cells of this highly vascularised region are arranged in irregular clusters and arcades. Cells of the *zona glomerulosa* are capable of regenerating cells of the *zona fasciculata* and *zona reticularis* via a progenitor cell population situated between the *zona glomerulosa* and *fasciculata* (Neville and O'Hare 1985; Teebken and Scheumann 2000).

The *zona fasciculata*, which lies between the *zona glomerulosa* and *zona reticularis*, comprises ± 75% of the total cortex volume. Cells in this region consist of columns of polyhedral cells, surrounded by straight capillaries angled at the same position as the cells. Cells in the *zona fasciculata* produce the glucocorticoids, cortisol and CORT, as well as trace amounts of dehydroepiandrosterone (DHEA) (Ehrhart-Bornstein et al. 1998; Young and Heath 2002).

The innermost region, the *zona reticularis,* encircles the medulla and consists of irregular branching cords and cell clusters separated by capillaries. The *zona reticularis* comprises ± 10% of the cortex volume and produces the adrenal androgen precursors, DHEA, dehydroepiandrosterone-sulfate (DHEAS) and A4 (Arlt and Stewart, 2005; Mescher, 2010).

3.3 Cholesterol as precursor for adrenal steroid hormone biosynthesis

Cholesterol, the common precursor for adrenal steroid hormone production, is mainly derived from circulating lipoproteins including LDL- and HDL cholesterol esters. Cholesterol can also be synthesized *de novo* from acetate in the endoplasmic reticulum of steroid producing cells or it can be mobilized from other intracellular sources. LDL cholesterol ester uptake is mediated by the LDL-receptor via receptor-mediated endocytosis. The endosome fuses with lysosomes containing acid lipase, which hydrolyses the LDL cholesterol to release free cholesterol. Cholesterol is subsequently transported to the endoplasmic reticulum where it is either used for adrenal steroid hormone production or converted to cholesterol esters by acyl-coenzyme A:cholesterol acyltransferase (ACAT) and stored in lipid droplets. These cholesterol esters can be converted back to free cholesterol by hormone-sensitive lipase (HSL) on demand (Faust et al. 1977; Brown et al. 1979; Gwynne and Strauss 1982; Mason and Rainey 1987; Ungewickell and Hinrichsen 2007; Rone et al. 2009;

Miller and Auchus 2011). HDL cholesterol ester uptake is mediated by the scavenger receptor class B, type-I receptor (SRBI), which forms a hydrophobic channel allowing for direct incorporation into the plasma membrane. HDL cholesterol esters are either converted to free cholesterol by HSL or stored in lipid droplets (Fig. 3.2) (Kraemer and Shen 2002; Connelly and Williams 2003; Rone et al. 2009). The transport of free cholesterol across the aqueous cytosol towards the outer mitochondrial membrane (OMM) is mediated by binding proteins which contain steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domains (Soccio and Breslow 2003; Miller and Auchus 2011). The transport of free cholesterol from the OMM to the inner mitochondrial membrane (IMM) by StAR is the rate-limiting step in adrenal steroid hormone production and is directly dependent on the availability of free cholesterol. At the IMM, P450 side chain cleavage (CYP11A1), also known as P450 scc, catalyses the conversion of cholesterol to PREG (Fig. 3.2).

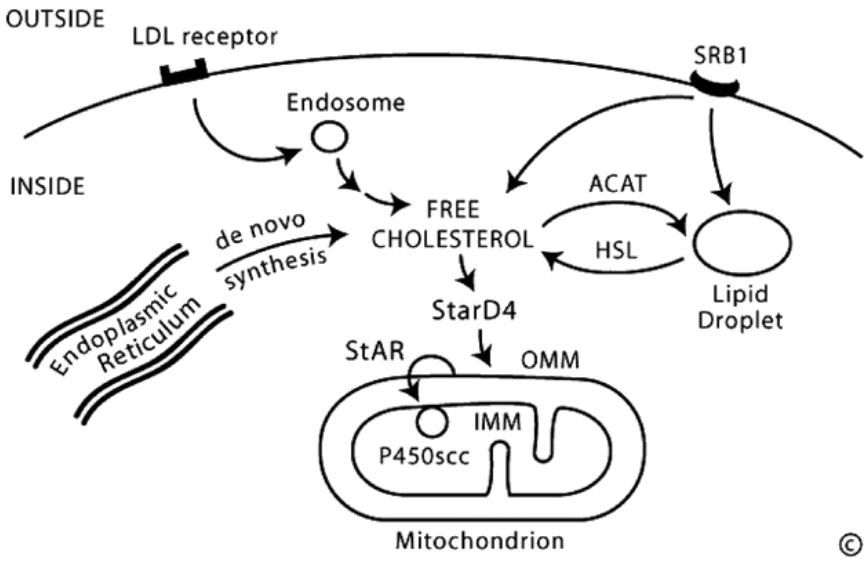


Figure 3.2: Principal pathways involved in intracellular cholesterol transport pathways. LDL, low-density lipoprotein; SRB1, scavenger receptor class B type-I; ACAT, acyl-coenzyme A:cholesterol acyltransferase; HSL, hormone-sensitive lipase; StAR, steroidogenic acute regulatory protein; StarD4, StAR-related lipid transfer domain; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; P450scc, cytochrome P450 side chain cleavage. Reproduced from Miller and Auchus (2011) with permission from Endocrine Society.

PREG subsequently enters the endoplasmic reticulum, where it serves as the precursor steroid metabolite to adrenal steroid hormone production through a network of enzyme-catalysed reactions. Adrenal steroid hormones therefore possess closely related chemical structures to that of cholesterol, based on the common cyclopentanoperhydrophenanthrene ring structure (You 2004) as shown in Figure 3.3.

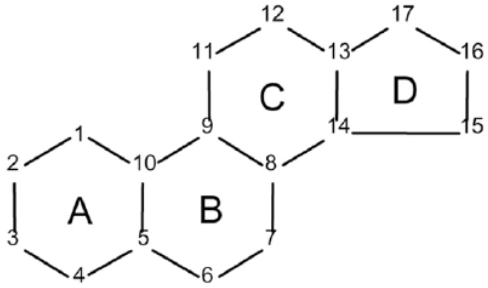


Figure 3.3: Cyclopentanoperhydrophenanthrene ring representing the backbone structure of all adrenal steroid hormones. Reproduced from You (2004) with permission from Elsevier.

3.4 Enzymes catalysing adrenal steroid hormone production

Adrenal steroid hormones are biosynthesized in the adrenal cortex from cholesterol via two distinct groups of enzymes - the P450 enzymes and the HSD enzymes. Adrenal P450 enzymes include CYP11A1, CYP17A1, CYP21A2, CYP11B1 and aldosterone synthase (CYP11B2), while the HSD enzymes include 3βHSD2, several isoforms of 17β-hydroxysteroid dehydrogenase (17βHSD) as well as 11βHSD1 and 11βHSD2 (Miller and Auchus, 2011). The 17βHSDs and 11βHSDs are, however, expressed at much lower levels compared to 3βHSD2 (Rege et al., 2013). Within the adrenal cortex, steroid production catalyzed by 3βHSD2, CYP17A1 and CYP21A2 occur in the endoplasmic reticulum, while reactions catalysed by CYP11A1, CYP11B1 and CYP11B2 occur in the mitochondria.

The mitochondrial enzymes require an electron shuttle system, adrenodoxin/adrenodoxin reductase, in order to hydroxylate or oxidize steroid

metabolites, while the enzymes located in the endoplasmic reticulum, also known as microsomal P450 enzymes, require electrons from NADPH via the enzymatic action of P450 oxidoreductase (POR) (Miller and Auchus, 2011). CYP17A1 lyase activity is dependant on an additional cofactor, namely cytochrome b_5 (Cyt-b_5), which is a flavoprotein functioning as an allosteric facilitator between CYP17A1 and POR (Storbeck et al. 2013).

P450 enzymes are functionally unidirectional, resulting in the accumulation of steroid products without driving the flux back to precursor steroid metabolites (Miller and Auchus 2011). Reactions catalysed by the HSDs, on the other hand, are reversible under certain *in vitro* conditions, with the reaction direction being dependent on substrate-, product- and co-factor availability. *In vivo*, however, the HSD enzymes catalyse one reaction only — either the oxidation or reduction of steroid metabolites (Agarwal and Auchus, 2005).

3.4.1 P450 enzymes

P450 enzymes are membrane bound proteins belonging to a superfamily of heme-containing proteins, and are present in all eukaryotic and some prokaryotic organisms. The “P450” annotation is derived from “pigment 450” due to the enzyme’s unique spectral property of absorbing light maximally at 450 nm when the reduced form of the enzyme is complexed with carbon monoxide *in vitro*. In addition, it was shown that P450-containing microsomes treated with detergent were converted to a solubilized form which, under reduced conditions and in the presence of carbon monoxide, resulted in an absorption maximum at 420 nm. This form of P450, referred to as “P420”, represented the inactive form of P450 (Miller and Auchus, 2011; Omura and Sato, 1962).

The P450 enzymes, also referred to as monooxygenases, function as strong oxidants since they catalyse oxidative reactions using molecular oxygen. P450 enzymes utilize NADPH as an electron donor to incorporate one oxygen atom into the substrate in the form of a hydroxyl group, while the other atom is reduced to water. The general P450 catalysed reaction can be written as follows:

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

A general mechanism for P450 catalysed hydroxylation reactions has been established over many years (Fig. 3.4) and consists of the following steps: Initially, the enzyme is in a resting low spin (LS) ferric (Fe^{3+}) state with a water molecule as the sixth ligand (1). Substrate (RH) binding in the active pocket displaces the water molecule and generates a high-spin (HS) substrate bound complex (2). The higher positive reduction potential of the HS Fe^{3+} complex leads to a single electron transfer from the redox partner, and the complex is subsequently reduced to the ferrous (Fe^{2+}) state (3). Molecular oxygen binds to the ferrous enzyme-substrate complex to form an oxygen-P450-substrate complex (4), which in turn results in the transfer of the second electron from the redox partner, leading to the formation of a Fe^{3+}-dioxo complex (5a). Protonation of this complex results in the formation of a peroxo-Fe^{3+} intermediate (5b), which undergoes a second protonation reaction, resulting in the splitting of molecular oxygen. One of the oxygen atoms are transferred to water, while the other atom remains bound to the ferric iron to form the reactive species (6), which subsequently transfers the distal oxygen atom to the substrate (7). Once the substrate is hydroxylated, it is released from the complex and replaced by water, regenerating the resting ferric state (1). In addition to various intermediate states, the P450 reaction cycle also involves three abortive reactions. The first reaction includes the autoxidation of the oxy-ferrous complex (4), with the subsequent production of a superoxide, while the enzyme returns to the resting state (2). The second reaction involves a peroxide shunt in which the hydroperoxide anion (5b) dissociates from the iron-producing hydrogen peroxide, while the enzyme returns to the resting state. Finally, an oxidase uncoupling can occur, in which the ferryl-oxo intermediate is oxidized, yielding water instead of the hydroxylated substrate (6) (Denisov et al. 2005).

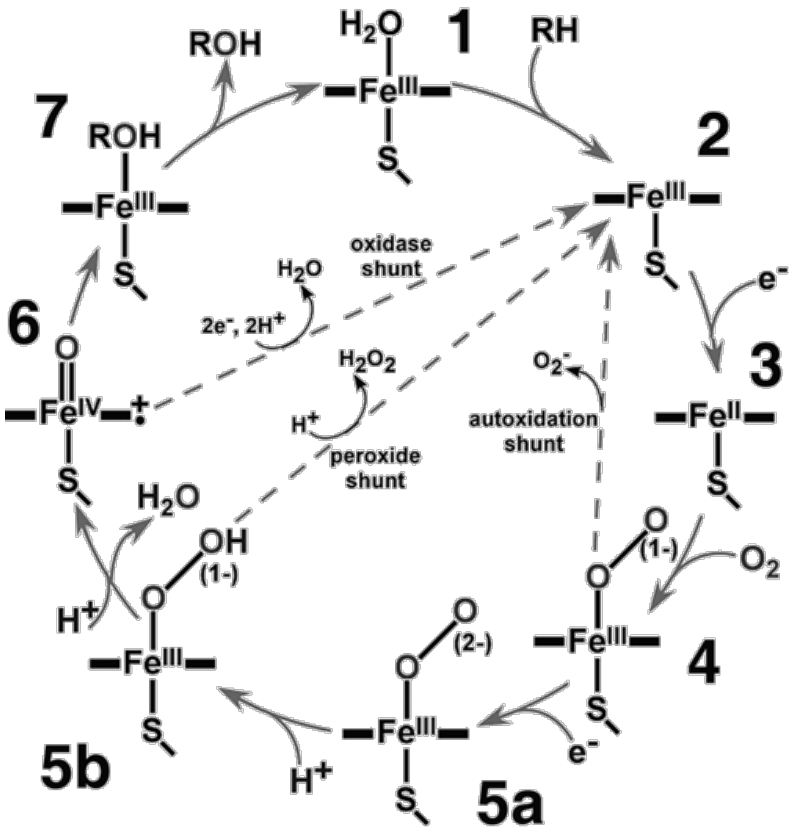


Figure 3.4: Cytochrome P450 reaction cycle. The substrate is indicated as RH and the hydroxylated product as ROH. Reprinted with permission from Denisov et al. (2005). Copyright 2005, American Chemical Society.

P450 enzymes are divided into different classes based on their intracellular localization and the mechanism by which they receive electrons. There are two classes of P450s involved in mammalian steroid hormone production, namely type-1 and type-2 P450 enzymes, which are localized in the mitochondria and endoplasmic reticulum, respectively. Type-1 mitochondrial enzymes (CYP11A1, CYP11B1 and CYP11B2) utilize NADPH as an electron donor, which transfers a high potential electron via the flavoprotein, adrenodoxin reductase, and then via adrenodoxin, a nonheme iron-sulfur protein. Adrenodoxin subsequently transfers the electron to the substrate via the P450 heme centre (Fig. 3.5A). Type-2 microsomal enzymes (CYP17A1, CYP21A2 and CYP19) receive electrons from NADPH via the single 2-flavin protein, POR. The electrons from NADPH are firstly transferred to a flavinadenine dinucleotide (FAD), secondly to flavinmononucleotide (FMN), and

finally to the substrate via the P450 heme centre (Fig. 3.5B) (Hannemann et al., 2007; Miller and Auchus, 2011; Payne and Hales, 2004).

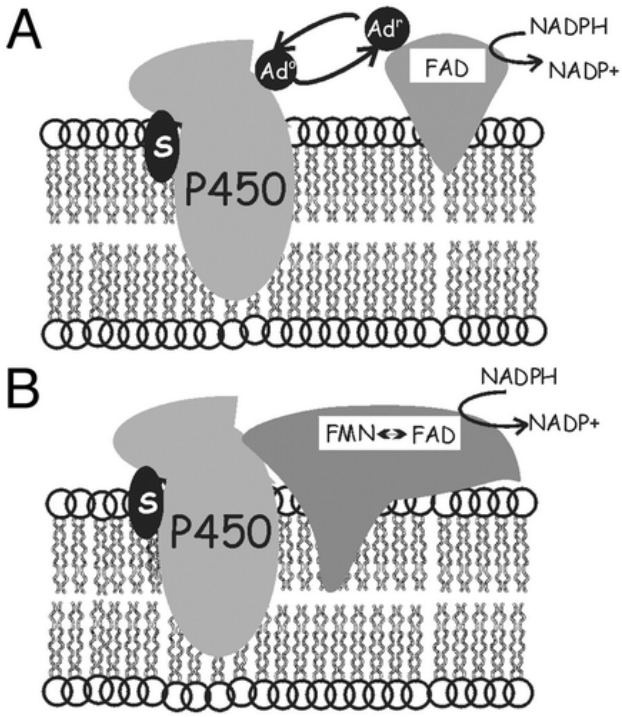


Figure 3.5: Schematic representation of mitochondrial (A) and microsomal (B) electron transfer systems. FAD, flavinadenine dinucleotide; Ad^r, adrenodoxin reductase; Ad^o, adrenodoxin; FMN, flavinmononucleotide; S, substrate. Reproduced from Payne and Hales (2004) with permission from Endocrine Society.

3.4.2 Hydroxysteroid dehydrogenases

The HSDs are non-metallic enzymes belonging to the short-chain alcohol dehydrogenase reductase superfamily. These enzymes catalyse the reduction and oxidation of steroid hormones and require NADH/NADPH as donors and NAD^+/$NADP^+$ as acceptors of reducing equivalents. There are three main types of HSDs that catalyse reactions within the adrenal cortex, namely the 3βHSDs, 17βHSDs and 11βHSDs. These enzymes have various isoforms, each being a

product of a different gene. Based on their physiological activities, HSDs can either be classified as dehydrogenases or reductases. Dehydrogenases utilize $NAD^+/NADP^+$ as cofactor, oxidizing hydroxy-steroids to keto-steroids, while the reductases mainly utilize NADH/NADPH to reduce keto-steroids to hydroxy-steroids. Although these enzymes can be bi-directional *in vitro*, they exhibit either reductase or dehydrogenase activity *in vivo*, with the direction being dependant on co-factor availability as well as the relative affinities of the HSDs for these cofactors (Agarwal and Auchus, 2005; Miller and Auchus, 2011; Penning, 1997).

In humans, two distinct isoforms of 3βHSD have been identified, namely 3βHSD1 and 3βHSD2, with only the latter isoform being expressed in the adrenal (Rhéaume et al. 1991). Within the adrenal, 3βHSD2 catalyses the conversion of the Δ^5-steroids, PREG, 17OH-pregnenolone (17OH-PREG) and DHEA to their respective Δ^4-steroid products, PROG, 17OH-PROG and A4, in two sequential reactions. Using PREG conversion by 3βHSD2 as an example, the first step involves a NAD^+ dependant dehydrogenation reaction, in which the C3 hydroxyl-group is converted to a keto-group, yielding a Δ^5-3-keto-intermediate and NADH, followed by a second reaction in which NADH subsequently activates the isomerisation of the Δ^5-3-keto-intermediate, yielding a Δ^4-3-keto-steroid product (Payne and Hales, 2004; Thomas et al., 2003) (Fig. 3.6).

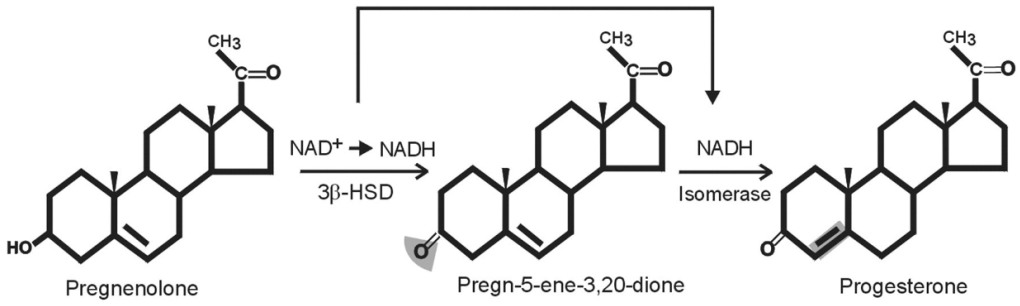


Figure 3.6: Enzymatic reactions catalysed by human 3βHSD2 in the adrenal using the conversion of pregnenolone (PREG) to progesterone (PROG) as an example. Reproduced from Payne and Hales (2004) with permission from Endocrine Society.

17βHSDs catalyse the inter-conversion between inactive 17-keto-steroids, such as DHEA, A4 and estrone to their respective active 17β-hydroxy-forms, androstenediol, testosterone and estradiol. There are at least 14 human 17βHSD isoforms, which all vary in terms of their sites of expression, substrate specificity, cofactor utilization and physiological functions (Miller and Auchus, 2011).

11βHSDs catalyse the inter-conversion between active glucocorticoids, (cortisol in humans and CORT in rodents) and their inactive keto-metabolites, (cortisone in humans and 11-DHC in rodents), respectively. There are two 11βHSD isoforms, *viz*. 11βHSD1 and 11βHSD2. 11βHSD1 is a NADPH-dependent microsomal oxo-reductase, which catalyzes the conversion of 11-DHC and cortisone, to their respective active glucocorticoids, CORT and cortisol (Cooper and Stewart, 2009). NADPH required for the oxo-reductase activity of 11βHSD1 is produced by the microsomal NADPH regenerating enzyme, hexose-6-phosphate dehydrogenase (H6PDH). H6PDH catalyzes the first two reactions of the pentose phosphate pathway to regenerate NADPH from $NADP^+$ via the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone within the endoplasmic reticulum lumen (Atanasov et al. 2008; Dzyakanchuk et al. 2009). H6PDH is an isomer of glucose-6-phosphate dehydrogenase (G6PDH), which catalyses the first step of the pentose phosphate pathway in the cytoplasm. In contrast to G6PDH, H6PDH does not only utilize G6P as substrate, but also other hexose-6-phosphates and sugars (Hino and Minakami 1982; Atanasov et al. 2008). In a study by Atanasov et al. (2004) it was shown that co-expresssion of 11βHSD1 and H6PDH in HEK-293 cells resulted in more than a 20-fold increase in the ratio of reductase:dehydrogenase activity of 11βHSD1 (Atanasov et al. 2004). The loss of NADPH production, as observed in H6PDH knockout mice, results in predominant dehydrogenase activity of 11βHSD1, which clearly indicates that H6PDH plays an indispensable role in 11βHSD1-depentant glucocorticoid production (Rogoff et al. 2007). *In vitro*, 11βHSD1 exhibits both dehydrogenase (oxidase) and oxo-reductase activity, due to the absence of H6PDH, and it is therefore able to catalyse the inter-conversion between active and inactive glucocorticoid metabolites. Co-transfection of 11βHSD1 and H6PDH changes the directionality of 11βHSD1 to mimic *in vivo* conditions in which the oxo-reductase activity of 11βHSD1 predominates. 11βHSD2, on the other hand, exhibits dehydrogenase activity only, and catalyses the conversion of the active glucocorticoids, cortisol and CORT to their respective inactive metabolites, cortisone

and 11-DHC (Fig 3.7). Besides from the inter-conversion between active glucocorticoids and their inactive keto-metabolites, we have recently shown that 11βHSD1 also catalyses the conversion of 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT) to 11OHA4 and 11β-hydroxytestosterone (11OHT), respectively, while 11βHSD2 catalyses the reverse reaction in which 11OHA4 and 11OHT is converted to 11KA4 and 11KT, respectively (Swart et al. 2013).

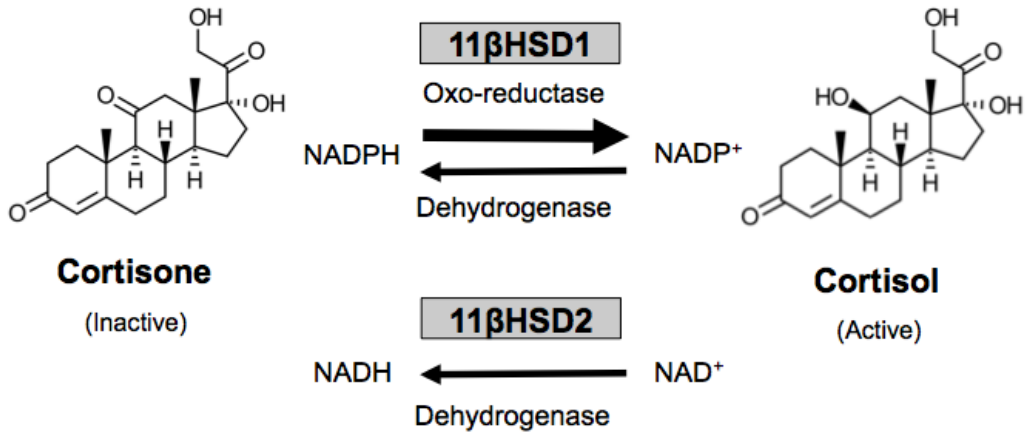


Figure 3.7: Inter-conversion between cortisol and cortisone by 11βHSD1 and 11βHSD2.

3.5 The adrenal steroidogenic pathway

The first step of adrenal steroidogenesis occurs in the mitochondria where cholesterol is converted to PREG by CYP11A1. The conversion of cholesterol to PREG involves three sequential reactions, which include the hydroxylation of C22 and C20 of cholesterol, followed by the cleavage of the C20-22 bond to yield PREG and isocaproaldehyde. PREG diffuses from the mitochondria to the endoplasmic reticulum, where it serves as a substrate for either CYP17A1 or 3βHSD2. Since CYP17A1 and 3βHSD2 are at the branch point in adrenal steroidogenesis, the relative catalytic activities and substrate specificities of these two enzymes toward their common substrates, PREG and 17OH-PREG, play a crucial role in the

determination of the steroid fluxes in the mineralocorticoid, glucocorticoid and adrenal androgen precursor pathways (Miller and Auchus, 2011).

CYP17A1 catalyzes two distinct reactions – the 17α-hydroxylation of PREG and PROG, yielding the glucocorticoid precursors, 17OH-PREG and 17OH-PROG, respectively; followed by the subsequent 17,20-lyase reaction in which the 17,20 bonds of 17OH-PREG and 17OH-PROG are cleaved to yield the adrenal androgen precursors, DHEA and A4, respectively. In humans, however, the 17,20-lyase reaction of CYP17A1 prefers 17OH-PREG as substrate and the conversion of 17OH-PROG to A4 is negligible. In addition, human CYP17A1 also hydroxylates PROG at C16, yielding 16OH-progesterone (16OH-PROG), a dead-end product.

As previously mentioned, 3βHSD2 catalyses the dehydrogenation of the Δ^5-steroids, PREG, 17OH-PREG and DHEA at C3 in two sequential reactions (Fig. 3.6) to yield their corresponding Δ^4-steroid products, PROG, 17OH-PROG and A4, which are the precursor metabolites for the mineralocorticoid-, glucocorticoid- and adrenal androgen precursor pathways, respectively. Cyt-b_5 is an electron transfer hemoprotein which influences the competition between CYP17A1 and 3βHSD2 by selectively promoting the 17,20-lyase activity of CYP17A1 by acting as an allosteric facilitator between CYP17A1 and POR. (Katagiri et al., 1995, 1982; Miller and Auchus, 2011). It was recently shown that Cyt-b_5 also augments the activity of 3βHSD by increasing the affinity of 3βHSD for NAD^+, which subsequently results in an increase of the rate limiting dehydrogenase reaction of this enzyme (Goosen et al. 2011, 2013). In addition to the activity of Cyt-b_5, phosphorylation of CYP17A1 serine/threonine residues has also been shown to promote the 17,20-lyase activity of CYP17A1 by facilitating the interaction between CYP17A1 and POR (Miller and Tee 2014).

CYP21A2 catalyzes the hydroxylation of PROG and 17OH-PROG at C21 to yield DOC and deoxycortisol, respectively, which are substrates for CYP11B2 and CYP11B1 in the mitochondria. CYP11B2, also known as aldosterone synthase, catalyzes three sequential reactions to yield the mineralocorticoid, ALDO. The first reaction involves the 11β-hydroxylation of DOC to yield CORT, which is hydroxylated at C18 to produce 18OH-corticosterone (18OH-CORT). CYP11B2 finally oxidizes the C18 hydroxyl group of 18OH-CORT, yielding ALDO. CYP11B1 catalyses the conversion of DOC and deoxycortisol to their respective glucocorticoids, CORT and cortisol, via hydroxylation at position 11β (Fig. 3.8).

We recently showed that both CYP11B1 and CYP11B2 catalyse the hydroxylation of A4 and testosterone, yielding 11OHA4 and 11OHT, respectively. These data suggests that CYP11B1 and CYP11B2 not only play a role in the biosynthesis of the mineralocorticoids and glucocorticoids, but also in the production of adrenal androgen precursors (Swart et al. 2013).

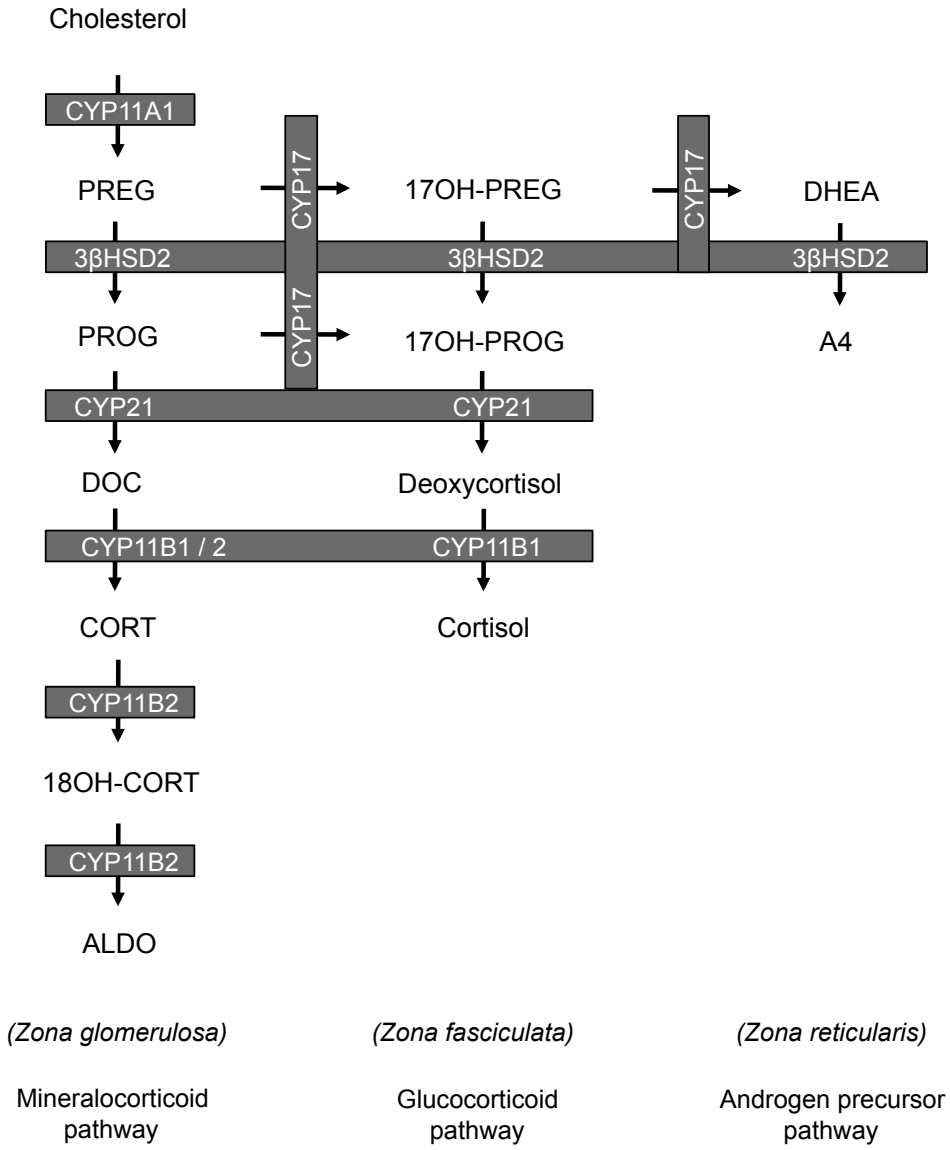


Figure 3.8: Major enzymes involved in human adrenal steroid hormone production.

As mentioned previously, the mineralocorticoids, glucocorticoids and adrenal androgen precursors are produced in three morphologically and biochemically distinct zones in the adrenal cortex, namely the *zona glomerulosa, zona fasciculata* and *zona reticularis,* respectively. Tissue specific expression of adrenal steroidogenic enzymes within these three zones, as well as their relative expression levels and catalytic activities, provide the biochemical basis for the differences in the steroid fluxes through the mineralocorticoid-, glucocorticoid- and adrenal androgen precursor pathways. Several enzymes are, however, expressed in more than one zone and defects in such enzymes can therefore affect the biosynthesis of various steroid hormones. Enzymes involved in the initial steps of adrenal steroidogenesis are expressed in all three zones of the adrenal cortex and they include StAR, CYP11A1, CYP21A2 and 3βHSD2, however, it has been shown that after the age of 8 years, the expression of 3βHSD2 in the *zona reticularis* decreases significantly. CYP17A1 is expressed in *zona fasciculata* and in the *zona reticularis*. The expression of Cyt-b_5 markedly rises in the *zona reticularis* after the age of 5 years and plateaus at the age of 13 years, whereas the levels in the *zona glomerulosa* and *zona fasciculata* remain extremely low at all ages. CYP11B2 is specific to the *zona glomerulosa* and CYP11B1 is specific to the *zona fasciculata* (Suzuki et al. 2000; Fallo et al. 2002; Enberg et al. 2003; Gomez-Sanchez et al. 2014; Rege et al. 2014).

Under physiological conditions, mineralocorticoid biosynthesis is regulated primarily by angiotensin II (Ang II), while glucocorticoid and adrenal androgen biosynthesis is regulated by ACTH. ACTH has been shown to significantly alter enzyme expression levels in the adrenal, which subsequently leads to altered steroid hormone production, influencing the steroid fluxes through the adrenal steroidogenic pathways.

The H295R cell line - a human adrenocortical carcinoma cell line, is an excellent model system for studying basal adrenal functions and the regulation of adrenal steroidogenesis (Rainey et al. 1994; Rehman et al. 2003). These cells are pluripotent and are capable of being directed to produce steroid metabolites from each of the three adrenal zones. H295R cells can be differentiated into ‘glomerulosa-like’ cells by pre-treating the cells with Ang II or potassium, which leads to increased ALDO production (Rainey et al. 2004). Pretreatment with forskolin- a diterpene which mimics the effects of ACTH via the activation of cAMP pathways, results in the

formation of ‘zona fasciculata-like’ cells, which leads to increased cortisol and adrenal androgen biosynthesis (Laurenza et al. 1989; Rainey et al. 1993).

In a study by Oskarsson et al. (2006), mRNA expression levels of steroidogenic enzymes were quantified in human adult adrenal glands as well as in H295R cells in the absence and presence of forskolin. Forskolin treatment in H295R cells significantly increased the expression levels of CYP11A1, CYP17A1, 3βHSD2, CYP21A2, CYP11B1, CYP11B2 and CYP19A1, with the most prominent increases being observed for CYP17A1 and CYP11B1, as their expression levels were upregulated between 5- and 6-fold following treatment. Interestingly, it was observed that the addition of forskolin changed the enzyme expression levels in the H295R cells to a steroidogenic transcript profile that was very similar to the profile of adult adrenal glands. Within the adult adrenal glands, CYP17A1 had the highest expression levels by far, followed by CYP11B1, StAR, CYP21A1, CYP11A1, 3βHSD2, SULT2A1, CYP11B2 and CYP19A1 (Oskarsson et al. 2006).

In a more recent study by Xing et al. (2011), the influence of ACTH on overall steroid production was assayed in adult adrenal primary cultures. ACTH treatment significantly increased the levels of the glucocorticoids, cortisol (63-fold), CORT (37-fold), the glucocorticoid precursor, deoxycortisol (23-fold), as well as the adrenal androgen precursors, A4 (26-fold), DHEA (18-fold) and 11OHA4 (17-fold), confirming the stimulation of both glucocorticoid and adrenal androgen precursor biosynthesis by ACTH (Xing et al. 2011).

3.6 Adrenal steroid hormones and their implications in disease

The adrenal steroid hormones — glucocorticoids, mineralocorticoids and adrenal androgen precursors are characterized by different physiological functions and as such, specific hormonal imbalances will impact uniquely on disease states. In the following sections, the three steroid hormone classes will be discussed in terms of their implications in disease. While the role of the adrenal androgen precursors and the mineralocorticoids will be discussed briefly, the main focus will be on the glucocorticoids.

3.6.1 Adrenal androgen precursors

Adrenal androgen precursors are C19 steroids produced in the *zona reticularis* of the adrenal cortex. The adrenal androgens precursors, DHEA and A4, only exhibit androgenic or estrogenic activity once converted by 3βHSD2, 17βHSD or CYP19 in peripheral target tissues to yield steroid metabolites such as testosterone and estrogen which are capable of activating steroid receptors. In women, roughly 75% of adrenal androgen precursors are utilized for peripheral estrogen production prior to menopause, while after menopause, it rises to 100%. In men, the contribution of adrenal androgen precursors is less significant (± 30-50%), since their primary source of androgens arise from the testes. DHEA secretion from the adrenal decreases over time and it is estimated that by the time women reach menopause, their DHEA secretion has decreased with about 60%. Due to significant inter-individual variation, some post-menopausal women have normal DHEA levels, while others have very low levels. There is no negative feedback mechanism controlling the levels of DHEA, and therefore, women with low DHEA levels will have a deficiency for the remainder of their lifespan. Since the adrenal is the sole producer of androgens in women, low post menopausal DHEA levels, together with the ageing process, leads to a number of medical conditions such as osteoporosis, muscle loss, fat accumulation, hot flashes, T2D, memory loss and possibly Alzheimer's disease. For most of these diseases, however, DHEA replacement therapy can be used to improve, correct, or even prevent some of these diseases. In men, on the other hand, adrenal androgen precursors have been implicated in prostate cancer, and both testicular- and adrenal androgen production need to be blocked in order to achieve optimal results during prostate cancer therapy (Arlt and Stewart, 2005; Labrie, 2010).

3.6.2 Mineralocorticoids

The principal mineralocorticoid, ALDO, is produced by CYP11B2 in the *zona glomerulosa* of the adrenal cortex and plays a key role in the regulation of sodium-, potassium- and water homeostasis. ALDO secretion is regulated by Ang II, potassium and, to a lesser degree, by ACTH. Ang II is produced via the RAAS, which regulates fluid and electrolyte balance. In response to low blood pressure, angiotensinogen is cleaved by renin to produce angiotensin I (Ang I), which has no

apparent biological activity. Ang I is subsequently converted by ACE to Ang II, a potent vaso-active peptide which cause blood vessels to constrict. Ang II increases the transcription of CYP11B2, stimulating the production of ALDO, which leads to increased sodium and water absorption, ultimately leading to an increase in blood pressure. Once the blood pressure has returned to normal, the secretion of renin is inhibited via a negative feedback mechanism (Ma et al. 2010; Beuschlein 2013).

Excessive production of ALDO is implicated in the development of various clinical conditions including heart failure (Güder et al. 2007; Tsutamoto et al. 2007), fibrosis of the heart (Brilla 2000), myocardial infarction (Beygui et al. 2006), renal failure (Tylicki et al. 2005) and hypertension (Vasan et al. 2004). Anti-hypertensive drugs include mineralocorticoid receptor (MR) antagonists, ACE inhibitors, CYP11B2 inhibitors and Ang II receptor blockers, amongst others (Hargovan and Ferro 2014). Spironolactone was the first MR agonist developed for the treatment of hypertension (Wolf et al. 1966). It was, however, shown to lack specificity for the MR, as it was able to also bind to progesterone and androgen receptors, resulting in a number of side effects including menstrual irregularities in women and sexual dysfunction in men (Parthasarathy et al. 2011). Another MR agonist, eplerenone, was subsequently developed, which had a reduced affinity for sex steroid receptors and was therefore less prone to cause sex hormone-related side effects. Both of these compounds have, however, been shown to cause electrolyte imbalances, in particular hyperkalemia, in up to 5% of patients (Weinberger et al. 2002). It was recently suggested that some of the adverse side effects of ALDO may occur through non-genomic pathways independent of MR stimulation. If non-genomic pathways contribute substantially to the effects brought about by ALDO, drugs that inhibit CYP11B2 might offer an advantage over MR antagonists by preventing the activation of both genomic and non-genomic pathways (Amar et al. 2013).

Since CYP11B2 catalyses the terminal steps in ALDO biosynthesis, selective inhibition of CYP11B2 is also of high pharmacological interest for the treatment of hypertension (Hakki and Bernhardt 2006; Baston and Leroux 2007; Schuster and Bernhardt 2007). However, selective inhibition of CYP11B2 has proved to be very challenging, since it shares 93% sequence identity with CYP11B1 (Curnow et al., 1991; Denner et al., 1995.; Kawainoto et al., 1990). Preclinical studies have shown that FAD286, a dextroenantiomer of fadrozole, significantly inhibits the activity of CYP11B2. FAD286 dose-dependently reduced the levels of ALDO, lowered blood

pressure and prevented end-organ damage. However, FAD286 was shown to lack selectivity for CYP11B2, since it also inhibited CYP11B1 to a large extent, which resulted in reduced cortisol biosynthesis. A second compound, LCI699 was subsequently developed and was the first orally active CYP11B2 inhibitor tested in clinical trials. Although LCI699 strongly and selectivity inhibited CYP11B2, it also inhibited CYP11B1 and impaired the plasma cortisol response to ACTH when administered at doses higher than 3 mg/day (Amar et al. 2013; Hargovan and Ferro 2014).

Due to the strong association between the RAAS and blood pressure regulation, blockade of this system is currently one of the key therapeutic targets for the treatment of hypertensive patients. Within this system, ACE inhibitors are currently the most clinically relevant pharmacological agents to block the activity of RAAS for the treatment of hypertension and CVD (Persson et al. 2010; van Vark et al. 2012). Numerous ACE inhibitors such as benazepril, catopril and perindopril have been developed and acute treatment with these ACE inhibitors was reported to significantly reduce circulating Ang II levels. Chronic treatment with ACE inhibitors, however, has been linked with the re-emergence of Ang II, which is referred to as “reactivation”, and it was shown to occur in one out of every six patients (Robles et al. 2014).

3.6.3 Glucocorticoids

The glucocorticoids, cortisol and CORT, are synthesised by CYP11B1 in the *zona fasciculata* of the adrenal cortex, which catalyses the conversion of DOC and deoxycortisol to CORT and cortisol, respectively (Miller and Auchus, 2011). In addition, cortisol and CORT are also regenerated from their respective inactive metabolites, cortisone and 11-DHC, via 11βHSD1 in peripheral tissues such as the liver, bone, adipose tissue, muscles and central nervous system (CNS). 11βHSD2 catalyses the inactivation of glucocorticoids and is expressed in mineralocorticoid target tissues such as the kidneys, colon, sweat and salivary glands, where it protects the MR from cortisol, which binds with a high affinity (Agarwal and Auchus, 2005; Cooper and Stewart, 2009; Tomlinson et al., 2004). Although both cortisol and CORT exhibit glucocorticoid activity, cortisol functions as the primary glucocorticoid in humans and it is produced in much higher levels than CORT. In rodents such as

rats and mice, however, CORT is the primary glucocorticoid due to the lack of CYP17A1 lyase activity (Miller and Auchus, 2011).

In circulation, 95% of glucocorticoids are bound to proteins – mainly to corticosteroid binding globulins (CBGs) and albumin, with only the free, unbound glucocorticoids having access to the glucocorticoid receptor (GR). Local mechanisms for the release of glucocorticoids from their carrier proteins include interactions with CBG receptors on the cell surface and enzymatic cleavage of CBGs (Golan et al. 2008).

Intracellularly, glucocorticoids exert their actions via the GR, however, glucocorticoids are also able to bind to the MR. While the GR is selective for glucocorticoids, the MR has equal affinities for the mineralocorticoid, ALDO and the glucocorticoids, cortisol and CORT. Since circulating cortisol levels are significantly higher than that of ALDO, the inactivation of cortisol to cortisone via 11βHSD2 in MR expressing tissues such as the kidney, plays a crucial role in protecting the MR from inappropriate activation by cortisol (Gathercole 2013).

Cortisol plays a vital role in the regulation of carbohydrate, protein and lipid metabolism; vascular responsiveness to catecholamines; maintenance of blood pressure; suppression of immune- and inflammatory responses; modulation of CNS function and regulation of the stress response. Cortisol regulates carbohydrate, protein and lipid metabolism in a coordinated fashion in order to increase circulating glucose levels at the expense of protein and fat stores. In the liver, cortisol promotes gluconeogenesis and glycogen synthesis, while simultaneously promoting protein catabolism in the muscle and lipolysis in adipose tissue in order to provide amino acids and glycerol for gluconeogenesis in the liver. In addition, cortisol reduces glucose utilization in tissues and decreases insulin sensitivity in adipose tissue. Low cortisol levels (hypocortisolism) is therefore characterised by low circulating glucose levels (hypoglycemia), while elevated cortisol levels (hypercortisolism) is characterised by high circulating glucose levels (hyperglycemia) (Costanzo 2014).

The biosynthesis and release of cortisol is under the control of the HPA axis - a complex system consisting of the hypothalamus, anterior pituitary and adrenal cortex. Interplay between these organs through the endocrine, paracrine and autocrine actions of their hormones is crucial for the maintenance of hormonal homeostasis. In response to stress, acute activation of the HPA axis results in the stimulation of the hypothalamus to secrete CRH and arginine vasopressin (AVP).

The role of AVP is to increase the activity of CRH, while also enhancing cardiovascular function, mood, memory and selective attention. CRH released from the hypothalamus stimulates the anterior pituitary to release ACTH, which in turn stimulates the adrenal cortex to release glucocorticoids. Increased glucocorticoid levels following ACTH stimulation have a negative feedback effect on CRH and ACTH in the hypothalamus and pituitary, respectively, acting to downregulate both the biosynthesis and secretion of these hormones in order to maintain homeostasis. In addition, ACTH is also able to downregulate the secretion of CRH from the hypothalamus (Tsigos and Chrousos 2002; Chung et al. 2011; Gathercole et al. 2013) (Fig. 3.9).

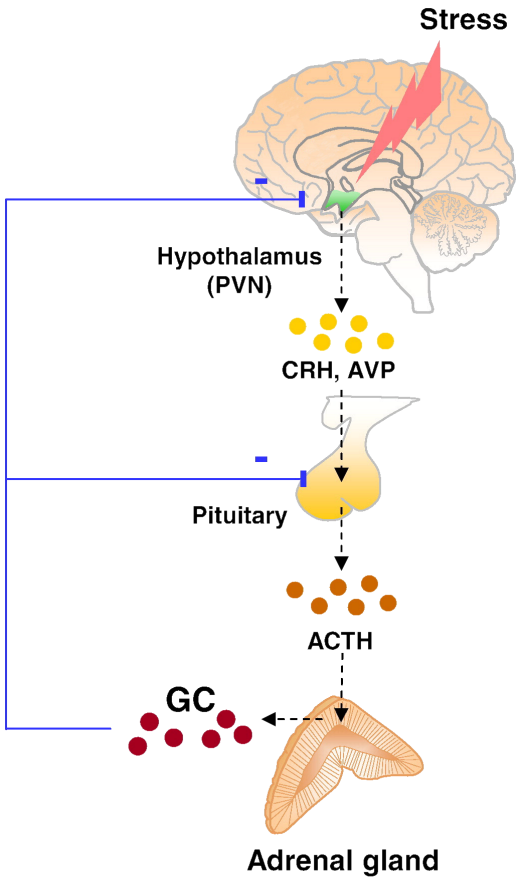


Figure 3.9: Schematic representation of the HPA axis. GC, glucocorticoids; PVN, paraventricular nucleus. Reproduced from Chung et al. (2011) with permission from Elsevier.

During chronic stress, the negative feedback regulatory mechanism is, however, overridden and circulating glucocorticoid levels remain elevated. Long term exposure to elevated glucocorticoid levels, either due to chronic stress, altered expression/activity of enzymes involved in glucocorticoid biosynthesis or abnormalities at receptor level, have been associated with hyperglycemia, hypertension, osteoporosis, suppression of the immune system, visceral obesity, insulin resistance, dyslipidemia, atherosclerosis and CVDs, which are characteristic of clinical conditions such as MetS and Cushing's syndrome (Chrousos and Gold 1998; Sen et al. 2008; Feelders et al. 2012; Gathercole et al. 2013). CVDs are currently the number one leading cause of death globally, with obesity, hypertension, diabetes and increased lipid levels being major contributing risk factors for the development of CVDs[5].

It is therefore evident that the maintenance of normal glucocorticoid levels is critical to sustain homeostasis and prevent disease. Strategies employed in the treatment of these metabolic diseases could thus be aimed at decreasing glucocorticoid levels either by inhibiting the enzymes involved in glucocorticoid biosynthesis (CYP11B1 and 11βHSD1) or antagonist activity at steroid receptor level (GR and MR).

Cushing's syndrome is a clinical phenotype induced by chronically elevated glucocorticoid levels. It shares almost all of the features of MetS, such as visceral obesity, T2D, dyslipidemia, hypertension, psychological dysfunction and osteoporosis. Cushing's syndrome is a rare disorder caused by a pituitary adenoma and is characterized by hyper-secretion of ACTH, which subsequently leads to excess glucocorticoid production (hypercortisolism) in the adrenal (Feelders et al., 2012; Gathercole et al., 2013). Of all the adverse systemic effects associated with hypercortisolism, cardiovascular associated complications are the most serious, and are one of the leading causes of death among patients suffering from Cushing's syndrome (Dekkers et al. 2007). The risk for CVDs in patients with Cushing's syndrome is aggravated due to obesity, T2D, dyslipidemia, insulin resistance and hypertension resulting from hypercortisolism (Arnaldi et al., 2003; Faggiano et al.,

[5] WHO, 2014 - Cardiovascular diseases: Fact sheet No.317.

http://www.who.int/mediacentre/factsheets/fs317/en/ (Updated March 2013)

2003; Feelders et al., 2012). The main difference between Cushing's syndrome and MetS is that Cushing's syndrome is associated with severe hypercortisolism, while patients with MetS do not necessarily have elevated circulating cortisol levels (Pasquali et al. 2006; Walker 2006; Gathercole and Stewart 2010).

The first-line treatment for Cushing's syndrome is surgery, with surgical remission being achieved for 65% - 100% of patients (Sudhakar et al. 2004; Prevedello et al. 2008). In contrast, individuals with comorbidities are twice as likely to have complications after surgery when compared with individuals without comorbidities (Patil et al. 2007). In severe cases, bilateral adrenalectomy offers a very effective treatment, provided that this is combined with permanent steroid replacement to prevent adrenal insufficiency (Smith et al. 2009). Other non-surgical treatments involve adrenal-blocking agents such as ketoconazole, metyrapone and mitotane. Although all of these agents lead to reduced cortisol secretion, none of these have been approved for the treatment of Cushing's syndrome since they do not selectively inhibit CYP11B1, resulting in a number of adverse side effects (Feelders et al., 2012). Ketoconazole, for example, has been shown to trigger toxicological effects *in vivo*, decreasing A4 and testosterone levels by up to 60% below basal plasma levels, while increasing 17OH-PROG levels. Metyrapone, on the other hand, has been reported to cause hypokalemia due to inhibition of both CYP11B1 and CYP11B2 (Nishizato et al. 2010; Emmerich et al. 2013).

As previously mentioned, CYP11B1 shares 93% homology with CYP11B2 and therefore, selective inhibition of these two enzymes is problematic. The compound, LCI699, selectivity inhibits CYP11B2 when administered at concentrations below 3 mg/day, while higher concentrations leads to significant inhibition of CYP11B1 (Amar et al. 2013; Hargovan and Ferro 2014). In a recent study by Bertagna et al. (2014), LCI699 (10-20 mg/day) strongly inhibited CYP11B1 and normalized urinary cortisol levels in patients with Cushing's syndrome during a 10-week proof-of concept study. Since patients with Cushing's syndrome generally have high blood pressure, the reduction of blood pressure by LCI699 was beneficial rather than harmful. In general, LCI699 was well tolerated in all patients involved in this study, and a large scale study of longer duration is currently being undertaken to investigate the safety and efficacy of LCI699 in the treatment of Cushing's syndrome (Bertagna et al. 2014).

Dysregulation of glucocorticoid action also plays a central role in the development of MetS, which refers to a cluster of abnormalities which include visceral obesity, dyslipidemia, hyperglycemia and hypertension (Obunai et al. 2007; Anagnostis et al. 2009; Athyros et al. 2010). MetS is associated with an increased risk for T2D, endothelial dysfunction, atherosclerosis, vascular morbidity and mortality (Lakka et al. 2002; Athyros et al. 2004; Kolovou et al. 2007; Obunai et al. 2007) and it is estimated that roughly one out of every four adults world-wide suffer from MetS (Athyros et al. 2005, 2007; Kolovou et al. 2007). Elevated 11βHSD1 expression in the liver and adipose tissue is one of the major causes of MetS and contributes substantially to the diabetic phenotype observed in *db/db* mice. Mice over-expressing 11βHSD1 in adipose tissue were shown to develop a 2-fold increase in intra-adipose glucocorticoid levels. Although plasma glucocorticoid levels remained unchanged, these mice nevertheless developed central obesity, hyperphagia, hyperinsulinemia and hyperglycemia (Masuzaki et al. 2001, 2003). Inhibition of 11βHSD1 has also been shown to have beneficial effects on glucose homeostasis and weight reduction in diabetic and obese mouse models (Alberts et al. 2002). 11βHSD1 knockout mice show reduced activation of key enzymes involved in gluconeogenesis such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). In addition, these mice were protected from hyperglycemia, dyslipidemia and obesity, despite consuming a high-fat diet (Kotelevtsev et al. 1997; Morton et al. 2004; Anagnostis et al. 2013). These findings were also reported in an earlier study by Dhanesha et al. (2012), in which inhibition of 11βHSD1 by carbenoxolone, a synthetic derivative of glycyrrhetinic acid, significantly affected obesity and glucose homeostasis in *db/db* mice, resulting in a dose-dependent improvement of energy expenditure, fat mass, serum lipid profiles, serum glucose tolerance and serum insulin tolerance. In addition, 11βHSD1 inhibition also reduced the activity of G6Pase and PEPCK in the liver, while improving glucokinase function in the pancreas and liver (Dhanesha et al. 2012).

Since MetS and Cushing's syndrome share numerous common features, with circulating glucocorticoid levels being significantly higher in patients with Cushing's syndrome, it has been proposed that increased glucocorticoid action at peripheral level may play a key role in the pathogenesis of these diseases. Intracellular glucocorticoid levels are not only determined by plasma levels, but also via the activity of 11βHSD1, especially in the liver and adipose tissue. 11βHSD1 plays a key

role in glucocorticoid metabolism at peripheral level and overexpression of this enzyme has been linked with MetS, central obesity and dysregulation of glucose and lipid metabolism (Anagnostis et al., 2013; Cooper and Stewart, 2009). Several potent and selective inhibitors of 11βHSD1 have been identified and include adamantyltriazoles (Olson et al. 2005), arylsulfonamidothiazoles (Barf et al. 2002) and anilinothiazolones (Yuan et al. 2007), amongst others. Some of the *in vivo* effects brought about by these compounds include reductions in plasma glucose levels, body weight, central obesity and adipocyte size, as well as improved insulin sensitivity and lipid profiles (Anagnostis et al. 2013). Selective inhibition of 11βHSD1 is crucial since non-selective inhibitors such as liquorice and its active metabolites, glycyrrhizic and glycyrrhetinic acids inhibit both 11βHSD1 and 11βHSD2, which result in an increased activation of the MR. In humans, ingestion of these compounds have been shown to result in "apparent mineralocorticoid excess" syndrome, hypertension encephalopathy, hypokalemia and suppression of the RAAS (Russo et al. 2000; Anagnostis et al. 2010, 2013; Pant et al. 2010).

Although circulating biologically active glucocorticoids, CORT and cortisol, are primarily derived from the adrenal gland via the activity of CYP11B1, glucocorticoids can also be regenerated from their inactive metabolites, 11-DHC and cortisone, via the activity of 11βHSD1 in peripheral tissues such as the liver and adipose tissue. Local glucocorticoid regeneration within these tissues have been shown to promote hepatic insulin resistance and fat accumulation (Masuzaki et al. 2001; Paterson et al. 2004). It is, however, still uncertain whether circulating glucocorticoid levels or glucocorticoids produced via 11βHSD1 in peripheral tissues plays the more prominent role in the development of MetS. These mechanisms were investigated in a recent study by Harno et al. (2013), in which the effect of 11-DHC administration was investigated in a liver-specific 11βHSD1 knockout (LKO) mouse model and compared to wild type (WT) mice. Following chronic administration of 11-DHC, WT mice had significantly increased circulating levels of CORT and displayed a MetS-like phenotype which included increased body weight gain, adiposity and the development of insulin resistance. In addition, reduced adrenal gland weight and decreased circulating ACTH levels in response to increased CORT levels suggest down-regulation of the HPA-axis. LKO mice, on the other hand, did not develop any of these adverse clinical side effects following administration of 11-DHC, despite having elevated circulating CORT levels, suggesting that 11βHSD1 activity in the

liver plays a central role in the development of insulin resistance and weight gain. The significant increase in CORT levels observed in LKO mice suggests that the major source of 11βHSD1-derived CORT that enters the circulation is generated elsewhere, most likely in the adipose tissue. Mice with complete deletion of 11βHSD1 did not have increased circulating CORT levels and showed no adverse metabolic effects following 11-DHC administration. Taken together, these data indicate that intracellular glucocorticoids regenerated in the liver via 11βHSD1, rather than circulating glucocorticoids, contribute towards the development of MetS. Selective inhibition of 11βHSD1 therefore seems like a promising target for the treatment of MetS (Harno et al. 2013).

3.7 Summary

From this chapter it is clear that adrenal steroid hormones play a crucial role in the maintenance of homeostasis within a variety of physiological processes, since abnormal steroid hormone production has been linked to numerous clinical conditions. Of particular interest for this study are the glucocorticoids, which if chronically elevated, have been linked to MetS and Cushing's syndrome, characterized by visceral obesity, insulin resistance, T2D, dyslipidemia, atherosclerosis, hypertension and CVDs. The maintenance of normal glucocorticoid levels therefore plays a central role in the prevention of these metabolic diseases. Strategies employed in the treatment of these disorders include, amongst others, inhibition of CYP11B1 and 11βHSD1. However, selective inhibition of CYP11B1 is difficult to achieve due to the enzyme's homology with CYP11B2. In contrast to compounds such as ketoconazole and metyrapone, the compound, LCI699, was shown to normalize urinary cortisol levels in patients with Cushing's syndrome without having any adverse side effects. Overexpression of 11βHSD1 plays a significant role in the development of MetS, and selective inhibition of 11βHSD1 has been reported to prevent hyperglycemia, dyslipidemia and obesity and to improve glucose homeostasis, lipid profiles and insulin tolerance.

In the next chapter, presented in the form of three published manuscripts, the influence of Rooibos and major Rooibos flavonoid compounds, which were assayed on the activities of key adrenal steroidogenic enzymes, CYP11B1, 3βHSD2,

CYP17A1, CYP21A2, 11βHSD1 and 11βHSD2, as well as on overall steroid production in H295R cells, under both basal and forskolin stimulated conditions, will be presented and discussed. In addition, the *in vivo* effects of Rooibos on circulating steroid hormone levels in experimental rats and in human test subjects at risk for developing CVD will be discussed.

CHAPTER 4

Published Manuscripts

4.1 Introduction

Chronically elevated glucocorticoid levels play a central role in the development of metabolic diseases. Numerous *in vivo* studies have reported that Rooibos consumption significantly improves glucose homeostasis, insulin resistance and lipid profiles, while also exhibiting potent anti-oxidant, anti-diabetic and cardio-protective properties. In addition, Rooibos has also been reported to modulate the activities of CYP17A1 and CYP21A2, two key enzymes involved in adrenal steroidogenesis. The aim of this thesis was therefore to determine the influence of Rooibos on adrenal steroid hormone production *in vitro* and *in vivo*, and in particular, on the levels of the glucocorticoids, cortisol and CORT.

The influence of an unfermented Rooibos extract and five of its major flavonoid compounds (the dihydrochalcones, aspalathin and nothofagin, their flavone analogues, orientin and vitexin and the flavonol, rutin) were firstly assayed on the activity of key adrenal steroidogenic enzymes - CYP17A1, 3βHSD2, CYP21A2 and CYP11B1, expressed in non-steroidogenic COS-1 cells. The influence of Rooibos and the aforementioned flavonoids on steroid hormone production was subsequently determined in H295R cells, a human adrenal cell model expressing the steroidogenic enzymes involved in the production of the mineralocorticoids, glucocorticoids and adrenal androgen precursors. Assays in H295R cells were conducted under both basal and forskolin stimulated conditions. Forskolin, a general inducer of adrenal steroidogenesis, was used to mimic the ACTH response in these cells, since H295R cells are insensitive to ACTH.

Since the 11βHSD isozymes play a crucial role in cortisol regeneration from the inactive metabolite, cortisone, the influence of Rooibos on the inter-conversion between cortisol and cortisone by 11βHSD1 and 11βHSD2 was investigated in CHO-K1 cells[6]. The influence of Rooibos on circulating glucocorticoid and testosterone levels was subsequently investigated *in vivo,* in male Wistar rats and in human

[6] CHO-K1 cells were used since COS-1 cells have endogenous 11βHSD2 activity

subjects (males and females) at risk for CVD. These studies are presented in the three manuscripts included in this chapter.

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The influence of *Aspalathus linearis* (Rooibos) and dihydrochalcones on adrenal steroidogenesis: Quantification of steroid intermediates and end products in H295R cells

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UPLC–MS/MS

ABSTRACT

The steroid hormone output of the adrenal gland is crucial in the maintenance of hormonal homeostasis, with hormonal imbalances being associated with numerous clinical conditions which include, amongst others, hypertension, metabolic syndrome, cardiovascular disease, insulin resistance and type 2 diabetes. *Aspalathus linearis* (Rooibos), which has been reported to aid stress-related symptoms linked to metabolic diseases, contains a wide spectrum of bioactive phenolic compounds of which aspalathin is unique. In this study the inhibitory effects of Rooibos and the dihydrochalcones, aspalathin and nothofagin, were investigated on adrenal steroidogenesis. The activities of both cytochrome P450 17α-hydroxylase/17,20 lyase and cytochrome P450 21-hydroxylase were significantly inhibited in COS-1 cells. In order to study the effect of these compounds in H295R cells, a human adrenal carcinoma cell line, a novel UPLC–MS/MS method was developed for the detection and quantification of twenty-one steroid metabolites using a single chromatographic separation. Under both basal and forskolin-stimulated conditions, the total amount of steroids produced in H295R cells significantly decreased in the presence of Rooibos, aspalathin and nothofagin. Under stimulated conditions, Rooibos decreased the total steroid output 4-fold and resulted in a significant reduction of aldosterone and cortisol precursors. Dehydroepiandrosterone-sulfate levels were unchanged, while the levels of androstenedione (A4) and 11β-hydroxyandrostenedione (11βOH-A4) were inhibited 5.5 and 2.3-fold, respectively. Quantification of 11βOH-A4 showed this metabolite to be a major product of steroidogenesis in H295R cells and we confirm, for the first time, that this steroid metabolite is the product of the hydroxylation of A4 by human cytochrome P450 11β-hydroxylase. Taken together our results demonstrate that Rooibos, aspalathin and nothofagin influence steroid hormone biosynthesis and the flux through the mineralocorticoid, glucocorticoid and androgen pathways, thus possibly contributing to the alleviation of negative effects arising from elevated glucocorticoid levels.

1. Introduction

In steroidogenesis, the steroid hormone output of the adrenal plays a pivotal role in the maintenance of hormonal homeostasis. Steroidogenic enzymes consisting of steroidogenic cytochrome P450 (P450) enzymes and 3β-hydroxysteroid dehydrogenase II (3βHSD) catalyse the biosynthesis of mineralocorticoids, glucocorticoids and androgen precursors in the adrenal cortex through multiple reactions (Fig. 1). Cytochrome P450 side-chain cleavage (CYP11A1) catalyses the conversion of cholesterol to pregnenolone (PREG) which is further metabolized by both 3βHSD and P450 17α-hydroxylase/17,20 lyase (CYP17A1) at a branch point in the pathway, thus determining the steroidogenic output of the adrenal. CYP17A1 catalyses the hydroxylation of PREG to yield 17-hydroxypregnenolone (17OH-PREG), which in turn is a substrate for the subsequent lyase reaction, yielding the androgen precursor dehydroepiandrosterone

Abbreviations: P450, cytochrome P450; 3βHSD, 3β-hydroxysteroid dehydrogenase II; CYP11A1, cytochrome P450 side-chain cleavage; PREG, pregnenolone; CYP17A1, P450 17α-hydroxylase/1720 lyase; 17OH-PREG, 17-hydroxypregnenolone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulfate; PROG, progesterone; 17OH-PROG, 17α-hydroxyprogesterone; 16OH-PROG, 16α-hydroxyprogesterone; A4, androstenedione; CYP21, cytochrome P450 21-hydroxylase; CYP11B1, cytochrome P450 11β-hydroxylase; CYP11B2, aldosterone synthase; ALDO, aldosterone; DOC, deoxycorticosterone; CORT, corticosterone; 11-DHC, 11-dehydrocorticosterone; 11βOH-A4, 11β-hydroxyandrostenedione; 11βHSD, 11β-hydroxysteroid dehydrogenases; ACE, angiotensin-converting enzyme; UPLC–MS/MS, ultra performance liquid chromatography/tandem mass spectrometry.

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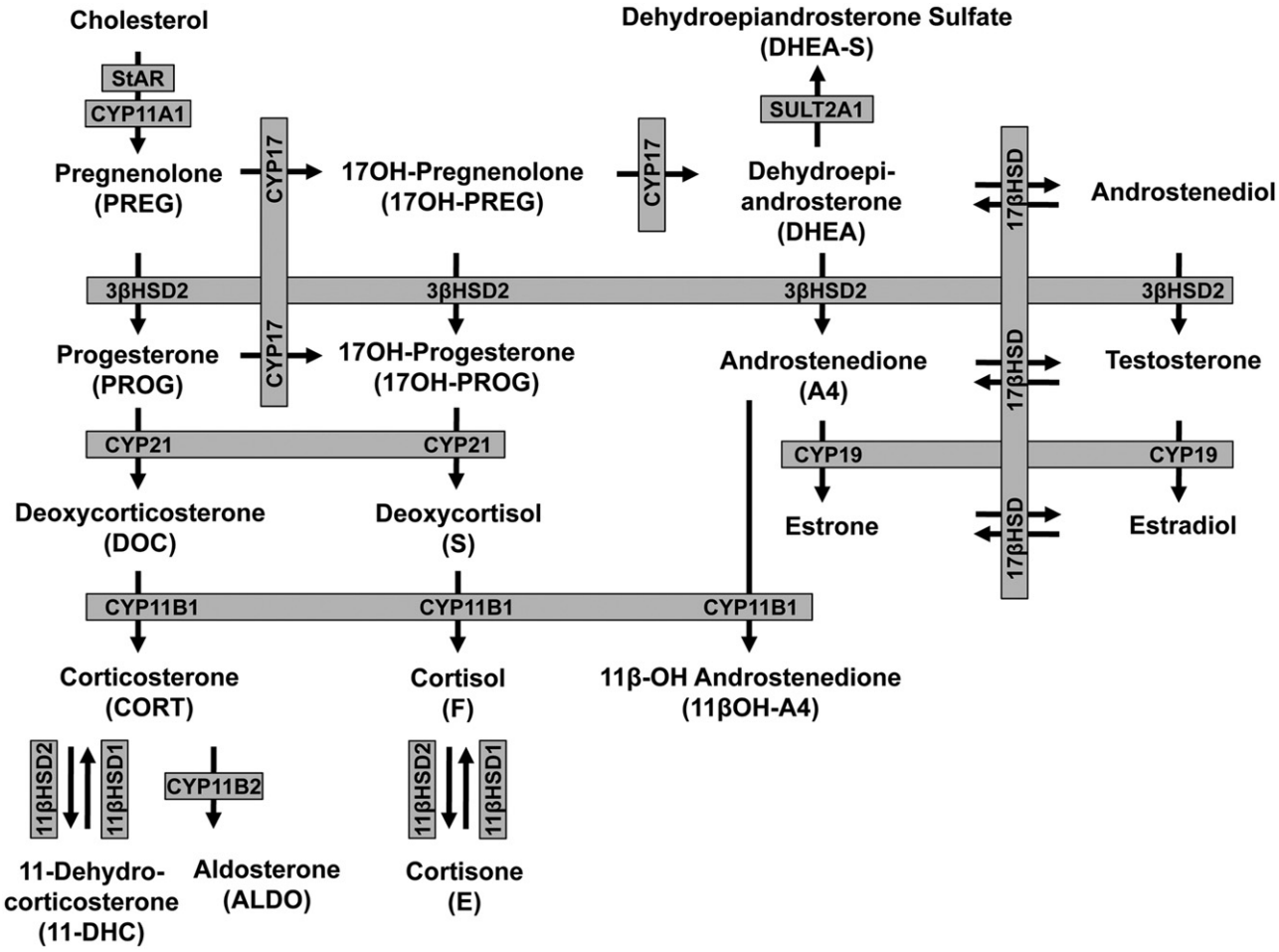

Fig. 1. Human steroid biosynthesis. Major enzymes and steroids involved in mineralocorticoid, glucocorticoid, androgen and estrogen biosynthesis.

(DHEA). In addition, human CYP17A1 converts progesterone (PROG) to 17α-hydroxyprogesterone (17OH-PROG) and 16α-hydroxyprogesterone (16OH-PROG) [1]. The metabolism of the Δ^5-steroids, PREG, 17OH-PREG and DHEA to the respective Δ^4-steroids, PROG, 17OH-PROG and androstenedione (A4), is catalysed by 3βHSD. Cytochrome P450 21-hydroxylase (CYP21) hydroxylates PROG and 17OH-PROG to yield the substrates for cytochrome P450 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). The latter catalyses the biosynthesis of the mineralocorticoid, aldosterone (ALDO), from deoxycorticosterone (DOC), while CYP11B1 catalyses the conversion of DOC and deoxycortisol to the glucocorticoids, corticosterone (CORT) and cortisol, respectively [2]. In addition to the major steroid hormones, the adrenal also produces, amongst others, sex steroids, albeit at low levels, as well as cortisone, 11-dehydrocorticosterone (11-DHC) and 11β-hydroxyandrostenedione (11βOH-A4), at significantly higher levels [3]. The former two metabolites are products of reactions catalysed by 11β-hydroxysteroid dehydrogenases (11βHSD type 1 and 2) [4], while the latter has been reported to be the product of the 11β-hydroxylation of A4 [5].

Glucocorticoids play a role in the maintenance of the homeostasis of the central nervous system, glucose homeostasis and immune modulation, and are considered stress hormones as their secretion is strongly altered by exposure to environmental stressors. Stress, be it psychological or physical, activates the hypothalamic–pituitary–adrenal (HPA) axis. Activation of the HPA-axis involves the release of adrenocorticotropic hormone (ACTH), resulting in the stimulation of the adrenal cortex with the subsequent elevation in circulating cortisol. Cortisol, released in response to stress, also forms part of the negative feedback system regulating the HPA-axis. During chronic stress, however, the negative feedback regulatory mechanism is overridden, resulting in elevated basal cortisol levels [6,7]. Elevated glucocorticoid levels, resulting from hormonal imbalances, have been associated with numerous clinical conditions which include, amongst others, hypertension, metabolic syndrome, cardiovascular disease, hyperglycemia, insulin resistance, type 2 diabetes, depressed immune function, osteoporosis and suppression of the reproductive system [7–10].

Maintaining normal cortisol levels is therefore critical to endocrine function. Therapies aimed at alleviating negative effects arising from the chronic stimulation of the HPA-axis could thus be aimed at decreasing plasma glucocorticoid levels. Strategies employed in the treatment of diseases associated with endocrine disorders involve selective inhibitors of either the enzymes catalysing adrenal steroid biosynthesis or antagonist activity at steroid receptor level. Several compounds have been identified which inhibit steroidogenesis by interfering with one or more enzymes in the steroidogenic pathway [11,12]. One such compound, ketoconazole, which is widely used to treat patients with Cushing's syndrome, inhibits CYP11A1 and CYP11B1 as well as ACTH secretion at therapeutic doses. Ketoconazole, however, has been found to trigger toxicological effects *in vivo*, decreasing A4 and testosterone levels to 60% below basal plasma levels while increasing 17OH-PROG levels. It is therefore critical in the development of pharmaceutical drugs to ensure that the inhibition of specific

steroidogenic enzymes is selective and that endocrine toxicity is avoided [13,14].

In recent years, the shift towards the use of natural and herbal medicinal products has led to attention being placed on the influence of, amongst others, polyphenolic compounds on steroidogenesis. Polyphenolic compounds include the dihydrochalcones, flavones, isoflavones and flavanols, and are commonly referred to as flavonoids. These secondary plant metabolites, abundantly present in soy and soy products, legumes and lentils, have been shown to demonstrate potent anti-oxidant, anti-inflammatory, anti-atherosclerotic and anti-mutagenic properties [15–19]. In addition, numerous studies have shown that flavonoid compounds exhibit phytoestrogenic activity and may aid clinical conditions such as osteoporosis, breast cancer and cardiovascular disease [20–22]. However, it has been suggested that flavonoid compounds may possibly be cytotoxic since they may act as mutagens, pro-oxidants and inhibitors of key enzymes [23]. High doses of genistein have, for example, been shown to result in decreased fertility and sexual dysfunction in experimental animals [24]. The overall health benefits of flavonoids therefore still remain uncertain [25]. The effect of flavonoid compounds on adrenal steroidogenesis has been investigated by various groups. Ohno et al. [26] showed that flavonoid compounds such as diadzein, genistein and 6-hydroxyflavone selectively inhibits key steroidogenic enzymes including 3βHSD, CYP17A1, CYP21 and CYP11B1 in H295R cells. Mesiano et al. [27] demonstrated that both genistein and diadzein inhibit cortisol production in ACTH-stimulated cultured fetal and postnatal adrenal cortical cells as well as in cAMP-stimulated H295 cells. More recently, Ohlsson et al. [28] showed that the flavonoids diadzein and genistein dose-dependently inhibit cortisol, ALDO and testosterone production in H295R cells.

Rooibos (*Aspalathus linearis*) is consumed world-wide as a herbal tea and is anecdotally reported to aid stress-related symptoms. It is a rich source of dietary polyphenols and as such, possesses potent antioxidant activities [29,30]. In addition, Rooibos extracts have been shown to exhibit anti-mutagenic, anti-cancer and immune modulating properties [31–33]. Rooibos consumption has been shown to significantly improve the lipid profiles and redox status in humans at risk for developing cardiovascular disease [34]. In addition, it has been shown by Persson et al. [35] that Rooibos significantly inhibits angiotensin-converting enzyme (ACE) activity after an oral intake of a single dose of Rooibos tea, supporting the potential role of Rooibos in the overall management of cardiovascular and metabolic-related diseases. Rooibos has been shown to have beneficial effects on glucose homeostasis and type 2 diabetes, with aspalathin stimulating glucose uptake in muscle tissues as well as insulin secretion from pancreatic β-cells [36]. Aspalathin is unique to Rooibos, while nothofagin has only been identified in one other species, *Nothofagus fusca* [37,38].

The aim of this study was to determine the influence of Rooibos and the dihydrochalcones, aspalathin and nothofagin, on the catalytic activities of CYP17A1 and CYP21 expressed in non-steroidogenic COS-1 cells, as well as on steroidogenesis in H295R cells, a human adrenal carcinoma cell line. Although previous studies have investigated the inhibitory effect of flavonoid compounds on adrenal enzyme activities, specific metabolites in the respective steroidogenic pathways, and on enzyme expression, the effect of these compounds on steroid hormone biosynthesis has not been fully addressed. Unsatisfactory analytical methods have impeded the efficient analyses and quantification of metabolites produced in adrenal steroidogenesis. In this study we report a novel ultra performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method for the identification and quantification of twenty-one steroid metabolites. This method enabled us to quantify not only the end metabolites of the mineralocorticoid, glucocorticoid and androgen precursor pathways, but also to simultaneously analyse the steroid profile of the intermediate metabolites in basal and forskolin stimulated H295R cells.

2. Materials and methods

2.1. Materials

Unfermented Rooibos was provided by the South African Rooibos Council. Aspalathin and nothofagin were supplied by Prof. W.C.A. Gelderblom (Medical Research Council, Western Cape, South Africa) Nucleobond® AX plasmid preparation kits were purchased from Machery-Nagel (Duren, Germany). COS-1 cells were obtained from the American Type tissue Culture Collection (Manassas, VA, USA) and *Mirus TransIT®*-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Penicillin–streptomycin, fetal calf serum and trypsin–EDTA were obtained from Gibco BRL (Gaithersburg, MD, USA). Deuterated cortisol (9,11,12,12-D4-cortisol) was purchased from Cambridge isotopes (Andover, MA, USA). Steroids, forskolin, trilostane, Dulbecco's modified Eagle's Medium (DMEM) and an MTT assay kit were purchased from Sigma–Aldrich (St. Louis, MA, USA). DMSO was obtained from Merck (Darmstadt, Germany). DMEM/F_{12} and gentamicin were purchased from Invitrogen/Gibco (Grand Island, New York, USA). Cosmic calf serum was supplied by HyClone®, Thermo Scientific (South Logan, Utah, USA). A bicinchoninic acid (BCA) protein determination kit was purchased from Pierce (Rockford, IL, USA). The UPLC BEH C18 column was purchased from Waters and the Kinetex PFP column was purchased from Phenomenex. All other chemicals were of the finest quality and supplied by trustworthy scientific supply houses.

2.2. Methanol extractions of unfermented Rooibos

Rooibos is produced both as a fermented and unfermented product, with the latter subjected to milder processes ensuring oxidative changes to secondary plant metabolites are minimized. A Rooibos extract was prepared by extracting 30 g unfermented plant material with 300 ml chloroform for 8 h using a glass soxhlet extractor fitted with a double wall condenser. The plant material was subsequently extracted with 300 ml methanol for 8 h. The extract was dried on a rotary evaporator and the vacuum released under nitrogen. The dried extract was resuspended in deionised water to a final concentration of 86 mg extract/ml and centrifuged at 6000 × *g* for 5 min. The supernatant was divided into aliquots and stored at −20 °C. The Rooibos extract was protected from light and oxygen at all times to avoid any compositional changes.

2.3. Enzyme assays in transiently transfected COS-1 cells

COS-1 cells were grown at 37 °C and 5% CO_2 in DMEM containing 0.9 g/l glucose, 0.12% $NaHCO_3$, 10% fetal calf serum and 1% penicillin–streptomycin. The cells were plated into 12 well dishes with each well containing 1×10^5 cells in 1 ml, 24 h prior to transfection. Cells were transiently transfected with 0.5 μg DNA (baboon CYP17A1/pCIneo, baboon CYP21/pCIneo and baboon CYP11B1/pTarget) and 1.5 μl *Mirus TransIT®*-LT1 transfection reagent according to the manufacturer's instructions. Control transfection reactions were performed using the pCIneo vector containing no DNA insert. Cells were incubated for 72 h after which the appropriate steroid substrate, PREG, PROG, 17OH-PROG, DOC, deoxycortisol or A4 was added to the medium. Substrate conversion in the presence of Rooibos was assayed by the addition of 50 μl extract (final concentration, 4.3 mg extract/ml). Aspalathin and nothofagin, dissolved in ethanol, were added to a final concentration of 10 μM. At specific time intervals, 500 μl aliquots were removed and the steroids extracted using a 10:1 volume of

dichloromethane to culture medium. The medium was removed and the dichloromethane phase dried under N_2. The steroids were resuspended in 150 μl methanol and analysed. After each experiment, the cells were washed and collected in phosphate buffer (0.1 M, pH 7.4), disrupted via sonication and the total protein content determined by the Pierce BCA method according to the manufacturer's instructions.

2.4. Steroid metabolism in H295R cells

H295R cells were grown to confluency at 37 °C and 5% CO_2 in growth medium, DMEM/F_{12}, supplemented with L-glutamine, 15 mM HEPES, pyridoxine, 1.125 g $NaHCO_3$/l, 1% penicillin streptomycin, 0.01% gentamicin and 10% cosmic calf serum, after which cells were plated into 12 well dishes (1 ml, 4×10^5 cells/ml). PREG metabolism was assayed as follows: after an incubation period of 48 h, the growth medium was aspirated off and replaced with growth medium containing 0.1% cosmic calf serum. Cells were incubated for a further 12 h after which the appropriate treatments were added to the medium. Basal steroid metabolism was assayed in the presence of Rooibos by the addition of 50 μl extract (final concentration, 1 mg extract/ml). Aspalathin and nothofagin were added to a final concentration of 10 μM. Basal steroid metabolism was stimulated with the addition of forskolin to a final concentration of 10 μM. A4 metabolism was assayed as follows: 24 h after plating cells the medium was replaced with growth medium containing 10 μM trilostane. After 24 h, the medium was replaced with growth medium containing 10 μM trilostane and 1 μM A4. Control assays included the addition of 10 μM trilostane and 1 μM cortisol; 10 μM trilostane; and medium only. After 48 h the medium (500 μl) was removed and the steroids extracted as described in Section 2.3. D4-cortisol (15 ng), was added to the dichloromethane/culture medium mixture as an internal standard prior to extraction.

2.5. Cell viability

2.5.1. COS-1 cells

The viability of the COS-1 cells was determined by investigating the effect of the test compounds on the ability of the cells to convert testosterone to A4 through endogenous 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2 activity [39]. Testosterone (1 μM) was added to confluent COS-1 cells in the absence and presence of the Rooibos extract (4.3 mg/ml), aspalathin (10 μM) and nothofagin (10 μM). After a 24 h incubation period the steroids were extracted as described in Section 2.3.

2.5.2. H295R cells

Confluent H295R cells were plated out in a 96 well plate (100 μl, 4×10^5 cells/ml) and incubated with the Rooibos extract (1 mg/ml), aspalathin (10 μM), nothofagin (10 μM) as well as forskolin (10 μM) for 48 h. Cell viability was subsequently assayed using a MTT toxicology assay kit according to the manufacturer's instructions.

2.6. Separation and quantification of steroid metabolites in COS-1 cells

Steroid metabolites were analysed by UPLC–MS/MS. Steroid metabolites resulting from the conversion of PREG, PROG, 17OH-PROG, deoxycortisol and A4 in COS-1 cells were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Waters UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm) column as previously described [40]. The mobile phases consisted of 1% formic acid (A) and acetonitrile (B). Steroids were eluted at a flow rate of 0.4 ml/min, using a linear gradient from 85% A to 80% B in 3.5 min, followed by a linear gradient from 80% B to 100% B in 0.1 min. The injection volume was 5 μl. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray in the positive ionization mode (ESI+). The following settings were used: capillary voltage of 2.8 kV, cone voltage 15–35 V, collision energy 4–32 eV, source temperature 100 °C, desolvation temperature 500 °C, desolvation gas 1000 l h^{-1} and cone gas 50 l h^{-1}. Calibration curves were constructed by using weighted ($1/x^2$) linear least squares regression. Data was collected with the MassLynx 4.0 software program.

2.7. Separation and quantification of steroid metabolites in H295R cells

2.7.1. Preparation of standards

Stock solutions of PREG, PROG, DOC, CORT, 18OH-CORT, ALDO, 11-DHC, 17OH-PREG, 17OH-PROG, 16OH-PROG, deoxycortisol, cortisol, cortisone, DHEA, DHEA-S, A4, 11βOH-A4, testosterone, DHT and β-estradiol were prepared in ethanol (2 mg/ml). Estrone was dissolved in acetone (2 mg/ml). A series of standards (0.0002, 0.002, 0.02, 0.1, 0.2, 1, 2 and 4 ng/μl) were prepared in methanol from the stock solutions. In addition, each standard contained an internal standard, D4-cortisol (final concentration, 0.1 ng/μl).

2.7.2. UPLC–MS/MS conditions

Steroid metabolites from conversion assays conducted in the H295R cells were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP (2.1 mm × 100 mm, 2.6 μm) column. The mobile phases consisted of 1% formic acid (A) and 49%:49%:2% methanol:acetonitrile: isopropanol (B). Steroid metabolites were eluted at a flow rate of 0.45 ml/min and the injection volume was set to 5 μl. Steroids were quantified using a Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) as described above. The gradient of the LC system is shown in supplementary Table 1, other relevant information including the parent and daughter ions, cone (V) and collision (eV) voltages, retention times and the validation of the UPLC–MS/MS assay is shown in supplementary Table 2.

2.8. Statistical analysis

All experiments were performed in triplicate and results are given as means ± SEM. Statistics were calculated by a one-way ANOVA, followed by a Dunnett's multiple comparison test using GraphPad Prism (version 5) software (GraphPad Software, San Diego, California). A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

The present study was undertaken to investigate the effect of Rooibos on adrenal steroidogenesis, and in particular, on glucocorticoid production. The influence of an unfermented Rooibos extract and two dihydrochalcone compounds, aspalathin and nothofagin, was investigated on two key adrenal steroidogenic enzymes, CYP17A1 and CYP21, by expression in non-steroidogenic COS-1 cells. We subsequently examined the effect of Rooibos, aspalathin and nothofagin on steroidogenesis in H295R cells.

3.1. Enzyme assays in COS-1 cells expressing CYP17A1 and CYP21

Both CYP17A1 and CYP21 play a central role in adrenal steroidogenesis with CYP17A1 catalysing the biosynthesis of androgen precursors while CYP21 is essential for the biosynthesis of mineralocorticoids and glucocorticoids. The effect of Rooibos (4.3 mg/ml),

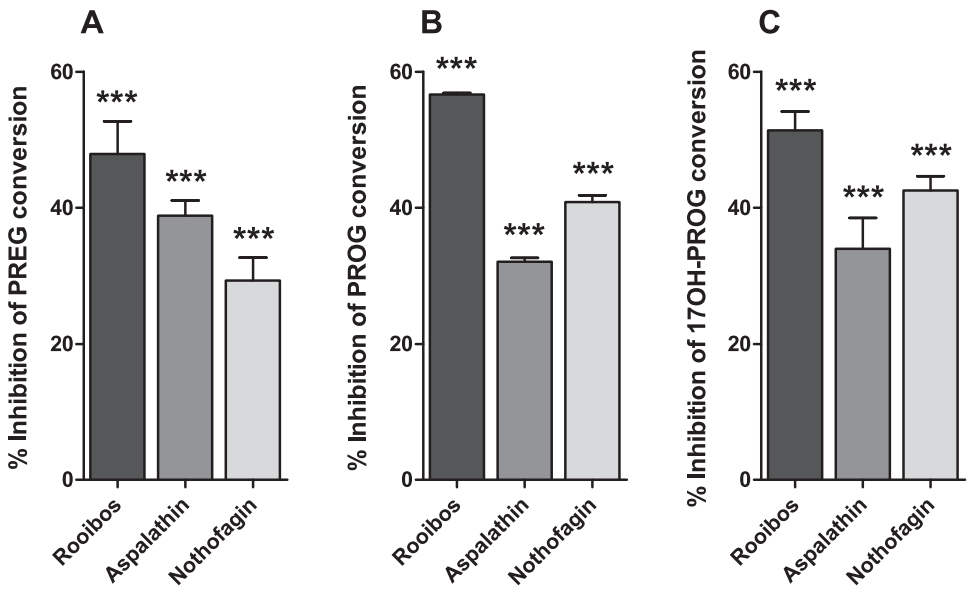

Fig. 2. The influence of Rooibos and dihydrochalcones on substrate conversion in transiently transfected COS-1 cells. (A) Inhibition of PREG (1 μM) conversion by baboon CYP17A1, (B) inhibition of PROG (1 μM) conversion by baboon CYP21 and (C) inhibition of 17OH-PROG (1 μM) conversion by baboon CYP21. Substrates were assayed in the presence of Rooibos (4.3 mg/ml), aspalathin (10 μM) and nothofagin (10 μM) after 4 h. Individual steroids were compared by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean ± SEM (***$P < 0.001$, $n = 3$).

aspalathin and nothofagin (10 μM) were assayed on CYP17A1 activity with PREG (1 μM) as substrate in non-steroidogenic COS-1 cells. After 4 h, PREG conversion was significantly inhibited in the presence of Rooibos (48%), aspalathin (39%) and nothofagin (29%) (Fig. 2A). The magnitude of CYP17A1 inhibition could significantly alter the flux through the steroidogenic pathway. Inhibition of CYP17A1 would likely result in decreased concentrations of glucocorticoids, deoxycortisol and cortisol, and androgen precursors, A4 and DHEA. Ohno et al. [26] previously found that formononetin, genistein and diadzein, at a concentration 25 μM, were unable to inhibit CYP17A1, while 6-hydroxy-flavone inhibited CYP17A1 significantly at the same concentration. Aspalathin and nothofagin are therefore more potent inhibitors, as significant inhibition of PREG conversion is observed at a lower concentration of 10 μM ($P < 0.001$).

The effect of Rooibos (4.3 mg/ml), aspalathin and nothofagin, (10 μM) on the catalytic activity of CYP21 expressed in COS-1 cells with both PROG (1 μM) and 17OH-PROG (1 μM) as substrates was also investigated. After 4 h, significant inhibition of PROG conversion was observed in the presence of Rooibos (57%), aspalathin (32%) and nothofagin (41%) ($P < 0.001$) (Fig. 2B). Similar results were obtained when 17OH-PROG was added as substrate, with Rooibos (51%), aspalathin (34%) and nothofagin (43%) inhibiting 17OH-PROG conversion significantly ($P < 0.001$) (Fig. 2C). In both assays, the presence of Rooibos showed the greatest inhibition, followed by nothofagin and aspalathin. Inhibition of CYP21 would inhibit the production of DOC and deoxycortisol, which in turn will lead to a decrease in the glucocorticoids, CORT and cortisol, as well as the mineralocorticoid, ALDO. Ohno et al. [26] previously demonstrated that the flavonoids 6-hydroxyflavone, diadzein and genistein significantly inhibit CYP21 at concentrations ranging from 12.5 to 25 μM ($P < 0.01$). Subsequent studies confirmed that genistein and diadzein are competitive inhibitors of both 3βHSD and CYP21 [26,41].

While CYP21 is unique to the adrenal and essential for mineralocorticoid and glucocorticoid biosynthesis, the dual catalytic activity of CYP17A1 and its competition for substrates with 3βHSD, places this enzyme in a pivotal position in determining the steroidogenic output of the adrenal cortex. Although the hydroxylation of PREG and the subsequent lyase reaction of the intermediate, catalysed by CYP17A1, were not assayed as individual reactions, the data clearly shows an inhibitory effect on the end products of the Δ^5-pathway. Both PREG and 17OH-PREG are substrates for 3βHSD which catalyses the biosynthesis of CYP21 substrates. PROG and 17OH-PROG are thus channelled into the mineralocorticoid and glucocorticoid pathways by CYP21. The role of 3βHSD, however, cannot be ignored. Ohno et al. [41] showed significant inhibition of 3βHSD by genistein, diadzein and quercetin at a concentration of 10 μM ($P < 0.01$). We found significant inhibition of PREG conversion by 3βHSD using the same concentration of aspalathin ($P < 0.01$) and nothofagin ($P < 0.001$) (data not shown).

The inhibitory effects observed on the catalytic activities of these steroidogenic enzymes by Rooibos and the two dihydrochalcone compounds at the tested concentrations was not due to the cells being compromised. The metabolism of testosterone was not impaired in the presence of the Rooibos extract (4.3 mg/ml), aspalathin (10 μM) or nothofagin (10 μM), confirming that the observed effects resulted from inhibition of the catalytic activities of the steroidogenic enzymes (data not shown).

3.2. Steroid metabolism in H295R cells

The H295R cell line is the first established cell line capable of producing all the steroids from the three adrenal cortex zones which include the mineralocorticoids, glucocorticoids and adrenal androgen precursors. In addition, these cells also express 17βHSD and aromatase (CYP19), and are thus capable of producing testosterone, estrone and β-estradiol, making this cell line an excellent model system for studying adrenal steroidogenesis [3,42]. The inter-conversion of the inactive keto-forms of estrogens and androgens to their respective active hydroxyl-forms are catalysed by various 17βHSD enzymes which vary in substrate specificity and tissue expression [43,44]. The accurate detection and quantification of the intermediates and end products in the steroidogenic pathways, however, still remains a challenge. In the most recent study to date, three different LCMS methods were used to identify the steroid metabolites produced in H295R cells [3]. In order to quantify the steroid metabolites of interest, we developed a novel UPLC–MS/MS method enabling the separation and quantification of twenty-one steroid metabolites, using a single chromatographic separation

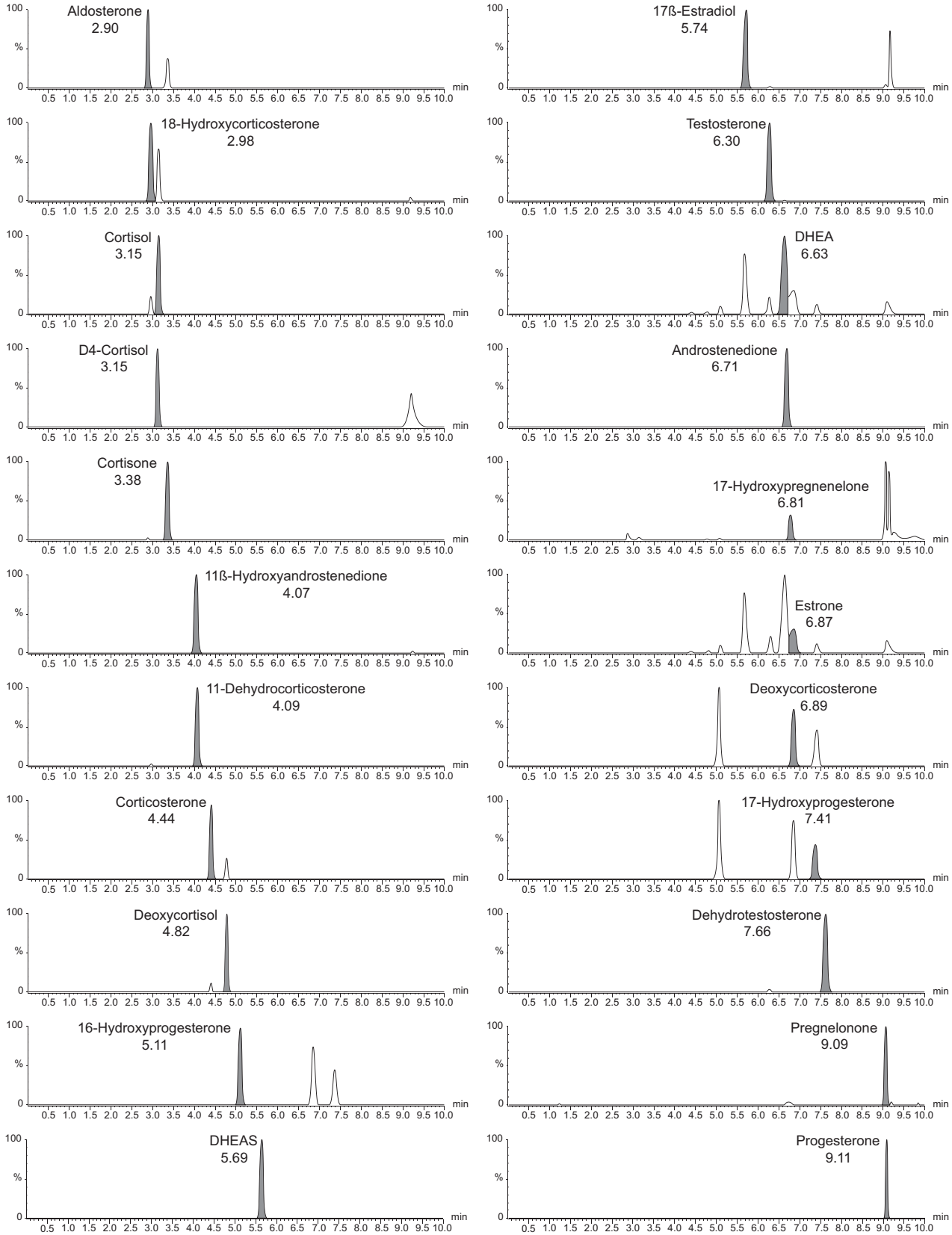

Fig. 3. UPLC–MS/MS chromatographic separation of 21 steroids. Chromatograms of steroid metabolites (5 μl each of a 2 μg/ml standard solution) and D4-cortisol are shown in multiple reaction monitoring (MRM) mode. Retention times of steroid metabolites (shaded peaks) are indicated on the chromatograms.

Table 1
Steroids produced in H295R cells. Cells were incubated in the absence and presence of forskolin (10 μM) for 48 h. Steroids were quantified by UPLC–MS/MS. Percentage of each steroid ± SEM was calculated by dividing the amount of individual steroid by the total steroid. Fold change ± SEM in response to forskolin treatment, was calculated from the changes in absolute values of individual steroids (not shown) compared to basal values. *P* values were calculated using a one-way ANOVA with a Dunnett's post test.

Steroid metabolite	Basal	+ Forskolin	
	% Total ± SEM	% Total ± SEM	Fold Change ± SEM
PREG	2.8 ± 1.7	0.7 ± 0.2	
PROG	1.2 ± 1.1	0.0 ± 0.0	
DOC	2.8 ± 0.2	3.0 ± 0.2	↑3.5 ± 1.1**
CORT	6.2 ± 0.5	18.6 ± 0.4	↑9.1 ± 2.0***
18OH-CORT	0.2 ± 0.0	0.9 ± 0.0	↑11.9 ± 2.8***
ALDO	0.1 ± 0.0	0.2 ± 0.0	↑14.1 ± 5.6***
11-DHC	0.3 ± 0.1	0.1 ± 0.0	
17OH-PREG	ND	ND	
17OH-PROG	1.5 ± 0.1	0.3 ± 0.0	
16OH-PROG	1.7 ± 0.0	0.6 ± 0.0	
Deoxycortisol	44.4 ± 1.4	24.6 ± 1.0	↑1.7 ± 0.5*
Cortisol	14.6 ± 1.0	34.2 ± 1.2	↑7.2 ± 1.9***
Cortisone	0.2 ± 0.1	0.1 ± 0.0	
DHEA	ND	ND	
DHEA-S	0.3 ± 0.2	0.0 ± 0.0	
A4	20.5 ± 0.3	12.5 ± 0.4	↑1.9 ± 0.6***
11BOH-A4	2.3 ± 0.1	3.5 ± 0.3	↑4.5 ± 0.7***
Testosterone	1.0 ± 0.1	0.5 ± 0.0	
DHT	ND	ND	
Estrone	ND	ND	
β-Estradiol	ND	ND	
Total steroid (nM)	3947	11141	↑2.8***

ND, not detectable ($n = 3$).
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

without prior derivatisation. The chromatographic separation of the steroid metabolites is shown in Fig. 3. The conditions and validations of this method are shown in supplementary material. This method enabled the detection and quantification of sixteen steroid metabolites from the medium of H295R cells after 48 h under basal conditions and in response to forskolin treatment. Forskolin was used as a general inducer of steroidogenesis since H295R cells are insensitive to ACTH [3]. Forskolin is a diterpene which mimics the effects of ACTH via the activation of adenyl cyclase (cAMP) pathways in adrenal cells [45]. While we did not detect DHEA, β-estradiol, estrone or 17OH-PREG, for which Xing et al. [3] detected low levels, we were however, able to detect and quantify ALDO, 11-DHC, cortisone, 16OH-PROG, 18OH-CORT and DHEA-S after 48 h under basal and forskolin-stimulated conditions in H295R cells.

Under basal conditions, the major steroid metabolites detected after 48 h were deoxycortisol (44.4%), A4 (20.5%), cortisol (14.6%) and CORT (6.2%) (Table 1). The higher concentration of deoxycortisol compared to that of cortisol is in accordance with data by Xing et al. [3] suggesting low expression of CYP11B1. However, in comparison, DOC levels are significantly lower than CORT levels, possibly due to metabolism of DOC by both CYP11B1 and CYP11B2. Forskolin stimulation significantly increased the production of ALDO (14.1-fold), 18OH-CORT (11.9-fold), CORT (9.1-fold), cortisol (7.2-fold), 11βOH-A4 (4.5-fold), DOC (3.5-fold), A4 (1.9-fold) and deoxycortisol (1.7-fold). After stimulation, cortisol (34.2%) was the most abundant steroid metabolite, followed by deoxycortisol (24.6%), CORT (18.6%) and A4 (12.5%). These results are in agreement with those obtained by Xing et al. [3] under similar experimental conditions. However, although cortisol and deoxycortisol were the most abundant metabolites under stimulated conditions, analyses of the steroid metabolites showed that there was a greater fold increase in the intermediates of the mineralocorticoid pathway, ultimately resulting in the 14-fold increase observed in ALDO levels.

In addition to altering the steroid profile of the cells, stimulation of forskolin resulted in a 2.8-fold increase in the total amount of steroid detected when compared to basal levels, with the total steroid concentration increasing from 4.0 μM to 11.1 μM (Table 1). It has previously been reported that the majority of agents that stimulate steroid hormone biosynthesis also upregulate steroid acute regulatory (StAR) protein expression. StAR is responsible for the transportation of cholesterol to the inner mitochondrial membrane where CYP11A1 metabolizes it to PREG [46]. King et al. [47] demonstrated that StAR mRNA levels increased by 260% after 24 h in the presence of 40 μM forskolin. Our data shows that the steroid flux through the glucocorticoid pathway is greater than the flux through the androgen precursor pathway under basal conditions, with even fewer metabolites being channelled through the mineralocorticoid pathway. Although the flux through the glucocorticoid pathway remains the highest upon forskolin stimulation, quantification of the intermediates shows that ALDO precursors are present in higher concentrations than that of the C19 metabolites.

Forskolin-stimulation also resulted in a significant increase in 11βOH-A4 production (0.09–0.39 μM) (Table 1). Rainey et al. [48] previously showed 11βOH-A4 to be one of the major steroids produced following forskolin stimulation. It is possible that 11βOH-A4 may be produced in the adrenal by either the lyase of cortisol or the hydroxylation of A4, with early studies favouring the hydroxylation of A4 [5,49]. *In vivo* and *in vitro* studies conducted by Axelrod et al. [50] showed A4 to be the major precursor of 11βOH-A4 in human and baboon adrenals. Conversion assays in human and baboon tissue showed that 1.3 and 1.4% ^{3}H-cortisol and 32 and 46% ^{3}H-A4 was incorporated into 11βOH-A4, respectively. Liakos et al. [51] later showed that transforming growth factor β1 (TGFβ1) inhibited the expression of CYP11B1 and CYP11B2 in H295R cells, resulting in a decrease in ALDO, cortisol and 11βOH-A4 production.

In this study we investigated the biosynthesis of 11βOH-A4 by H295R cells in the absence and presence of trilostane (10 μM), a selective inhibitor of 3βHSD. Neither A4 nor 11βOH-A4 was detected after 48 h in the presence of trilostane. The addition of A4 (1 μM) together with trilostane (10 μM) resulted in a significant increase in 11βOH-A4 levels, indicating that this metabolite is a product of the 11β-hydroxylation of A4 (Fig. 4). No 11βOH-A4 was detected following the addition of cortisol (1 μM) together with trilostane (10 μM). In addition, we assayed the conversion of cortisol in COS-1 expressing recombinant human CYP17A1, in the absence and presence of cytochrome b_5, which augments the lyase reaction, and did not detect 11βOH-A4 after 8 h by UPLC MS/MS analyses (data not shown). While the conversion of A4 to 11βOH-A4 by CYP11B1 has been demonstrated for non-primate species [52,53], to our knowledge, the 11β-hydroxylation of A4 by primate CYP11B1 has only been indirectly implied. We therefore assayed the metabolism of A4, deoxycortisol and DOC in COS-1 cells transiently co-transfected with baboon CYP11B1 and human adrenodoxin (ADX). The data clearly shows that CYP11B1 catalyses the conversion of A4 to 11β-OHA4 with negligible substrate remaining after 8 h (Fig. 5). In the assay in which both deoxycortisol (1 μM) and A4 (1 μM) were added together, the substrates did not appear to inhibit the conversion of each other after 8 h, although the rate of the conversions are yet to be determined. A conversion profile similar to that of deoxycortisol and A4 was obtained when DOC was added as substrate, with negligible substrate remaining after 8 h (results not shown). From our data it is clear that the production of 11βOH-A4 observed in the H295R cells could be attributed to the conversion of A4 by CYP11B1. Furthermore, the 4.5-fold increase observed in 11βOH-A4 levels under forskolin-stimulated conditions (Table 1) is therefore

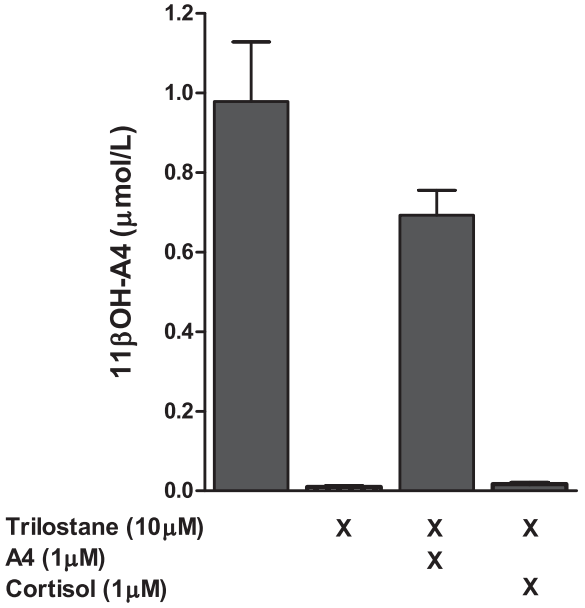

Fig. 4. Analysis of 11βOH-A4 production in H295R cells. Basal 11βOH-A4 production was assayed in the absence and presence of trilostane (10 μM) while the conversion of A4 (1 μM) and cortisol (1 μM) to 11βOH-A4 was assayed in the presence of trilostane after 48 h. Results are expressed as the mean ± SEM ($n = 3$).

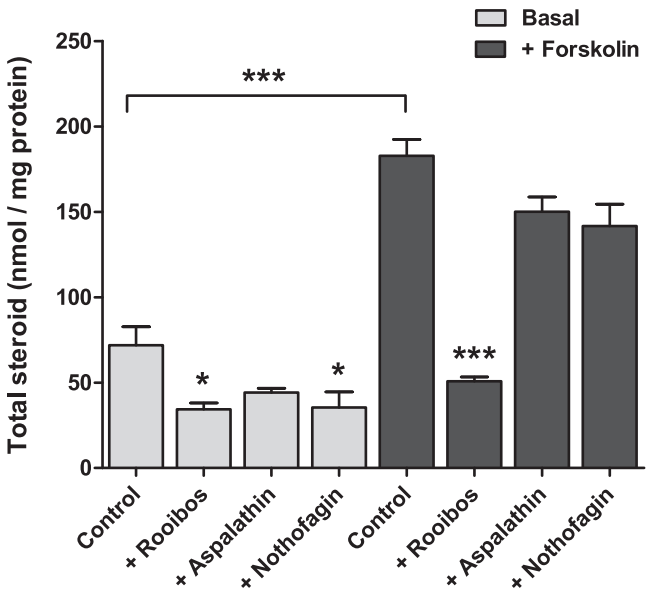

Fig. 6. Basal and forskolin-stimulated steroid production in H295R cells. Steroids were assayed with and without forskolin (10 μM) stimulation, in the presence of Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM) after 48 h. Data was analysed by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean ± SEM ($^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$).

to be expected since forskolin upregulates not only CYP11B1 mRNA but also the hydroxylase activity in H295R cells [51,54]. In addition, the significant increase in A4 levels in the H295R cells suggests that CYP17A1 activity was stimulated in the presence of forskolin, as was previously reported by Rainey et al. [48], thereby increasing the precursor metabolite for the biosynthesis of 11βOH-A4.

3.2.1. *The influence of Rooibos and flavonoids on H295R metabolism*

Since the goal of this study was to investigate the effect of Rooibos on the outcome of adrenal steroidogenesis, and in particular, on glucocorticoid production, PREG metabolism was investigated in H295R cells. We assayed the effect of Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM) on both basal and forskolin (10 μM) stimulated steroid metabolism. The total amount of steroid detected, decreased significantly under basal conditions in the presence of Rooibos (2-fold), aspalathin (1.6-fold) and nothofagin (2-fold) (Fig. 6). Similarly, under forskolin stimulated conditions, treatment with Rooibos, aspalathin and nothofagin decreased the total amount of steroid detected by 4.0-, 1.4- and 1.4-fold, respectively (Fig. 6). MTT assays conducted in the presence of 1 mg/ml Rooibos extract, 10 μM dihydrochalcone compounds and 10 μM forskolin showed that the inhibitory effects observed in the H295R assays were not due to a decrease in cell viability (data not shown). The greater inhibition observed by the addition of the Rooibos extract in the presence of forskolin may be attributed to compounds which could affect upstream processes. Genistein together with diadzein, have been shown to inhibit ACTH-stimulated cortisol production in cultured fetal and postnatal adrenal cortical cells at concentrations ranging from 0.4 to 40 μM. Both genistein and diadzein also inhibited cAMP-stimulated cortisol synthesis in H295 cells [27].

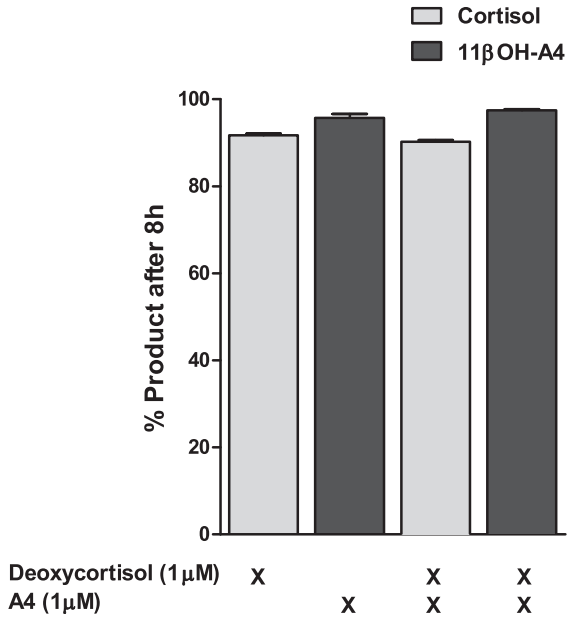

Fig. 5. Cortisol and 11βOH-A4 formation in transiently transfected COS-1 cells. Conversion of deoxycortisol and A4 was assayed after 8 h in COS-1 cells co-expressing baboon CYP11B1 and human ADX. Results are expressed as the mean ± SEM ($n = 3$).

It is possible that the decrease in steroid production by Rooibos may be due to the inhibition of either CYP11A1 or the transport of cholesterol to the inner mitochondrial membrane. We have shown that Rooibos, aspalathin and nothofagin significantly inhibit CYP17A1 and CYP21 in COS-1 cells and inhibition of these key enzymes would ultimately influence the steroid outcome of the adrenal gland. Under basal conditions, the addition of Rooibos and the dihydrochalcone compounds resulted in a small increase in the production of the mineralocorticoid, ALDO (Table 2). Significant reductions were, however, observed in the levels of A4 (Rooibos, 2.7-fold; aspalathin, 2.4-fold and nothofagin, 3.5-fold) and testosterone (Rooibos, 8.8-fold; aspalathin, 4.3-fold and nothofagin, 7.1-fold). The reduction of A4, and consequently of testosterone, is most likely due to inhibition of 3βHSD as human CYP17A1 does not readily catalyse the lyase of 17OH-PROG to A4. In addition, increased DHEA-S production (1.6-fold) was observed in the presence of Rooibos, which could also imply inhibition of 3βHSD. The increase in PREG and 17OH-PROG levels in the presence of

Table 2
Steroids produced in H295R cells under basal conditions in the presence of Rooibos and dihydrochalcones. Cells were incubated for 48 h with Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM). Steroids were quantified by UPLC–MS/MS. Percentage of each steroid ± SEM was calculated by dividing the amount of individual steroid by the total steroid. Fold change ± SEM in response to Rooibos, aspalathin and nothofagin treatment, was calculated from the changes in absolute values of individual steroids (not shown) compared to basal values. *P* values were calculated using a one-way ANOVA with a Dunnett's post test.

Steroid metabolite	Basal	+ Rooibos		+ Aspalathin		+ Nothofagin	
	% Total ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM
PREG	2.8 ± 1.7	5.4 ± 1.7		2.0 ± 0.4		0.9 ± 0.5	
PROG	1.2 ± 1.1	0.5 ± 0.1		0.2 ± 0.0		0.3 ± 0.1	
DOC	2.8 ± 0.2	2.8 ± 0.1		9.0 ± 0.8		11.6 ± 1.5	
CORT	6.2 ± 0.5	7.7 ± 0.4		10.4 ± 0.6		11.3 ± 1.0	
18OH-CORT	0.2 ± 0.0	0.7 ± 0.3		0.6 ± 0.1		0.7 ± 0.2	
ALDO	0.1 ± 0.0	0.3 ± 0.0		0.2 ± 0.0		0.2 ± 0.0	
11-DHC	0.3 ± 0.1	0.5 ± 0.0		0.2 ± 0.0		0.3 ± 0.0	
17OH-PREG	ND	ND		ND		ND	
17OH-PROG	1.5 ± 0.1	4.5 ± 0.1	↑1.6 ± 0.4*	0.7 ± 0.2	↓3.6 ± 1.1**	0.7 ± 0.2	↓4.8 ± 1.3**
16OH-PROG	1.7 ± 0.0	0.7 ± 0.1	↓5.0 ± 0.7***	1.2 ± 0.0	↓2.3 ± 0.4***	1.0 ± 0.1	↓3.6 ± 0.7***
Deoxycortisol	44.4 ± 1.4	27.2 ± 1.6	↓3.5 ± 0.6**	44.3 ± 0.3		42.4 ± 0.6	↓2.3 ± 0.6*
Cortisol	14.6 ± 1.0	23.8 ± 2.0		14.8 ± 0.1		15.3 ± 1.4	
Cortisone	0.2 ± 0.1	0.3 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	
DHEA	ND	ND		ND		ND	
DHEA-S	0.3 ± 0.2	0.5 ± 0.1	↑1.6 ± 0.7**	0.1 ± 0.0		0.1 ± 0.0	
A4	20.5 ± 0.3	16.3 ± 0.4	↓2.7 ± 0.6**	13.9 ± 0.9	↓2.4 ± 0.4**	13.0 ± 0.4	↓3.5 ± 0.8***
11BOH-A4	2.3 ± 0.1	8.7 ± 0.4	↑1.9 ± 0.4**	1.8 ± 0.1		1.7 ± 0.2	↓3.0 ± 0.6*
Testosterone	1.0 ± 0.1	0.3 ± 0.0	↓8.8 ± 2.0***	0.4 ± 0.1	↓4.3 ± 1.0***	0.3 ± 0.0	↓7.1 ± 2.6***
DHT	ND	ND		ND		ND	
Estrone	ND	ND		ND		ND	
β-Estradiol	ND	ND		ND		ND	
Total steroid (nM)	3947	1890	↓2.1*	2429	↓1.6	1952	↓2.0*

ND, not detectable ($n = 3$).
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Rooibos under stimulated conditions, suggest the inhibition of 3βHSD, CYP17A1 and CYP21. 16OH-PROG, a dead end product, was also reduced in the presence of Rooibos, suggesting either a reduction of PROG levels due to 3βHSD inhibition or inhibition of CYP17A1. Under basal conditions, cortisol and cortisone levels were not significantly affected, while basal deoxycortisol levels decreased significantly in the presence of Rooibos (3.5-fold) and nothofagin (2.3-fold), possibly due to inhibition of CYP21. Under basal conditions, both aspalathin and nothofagin significantly reduced 17OH-PROG, 16OH-PROG, A4 and testosterone levels, confirming our findings in COS-1 cells.

The effect of Rooibos was notably more pronounced during forskolin treatment than under basal conditions. Rooibos significantly decreased the levels of the glucocorticoids, cortisol (4.9-fold), cortisone (5.2-fold) and CORT (5.2-fold), as well as the glucocorticoid precursors, deoxycortisol (5.1-fold) and DOC (3.4-fold) under stimulated conditions (Table 3). Aspalathin and nothofagin also decreased cortisol levels by 1.3-fold and 1.7-fold, respectively. While no effect was observed in ALDO levels in the presence of Rooibos, there was a significant reduction of the precursor metabolites. Androgen precursor production was significantly affected by Rooibos, aspalathin and nothofagin under stimulated conditions. A4, testosterone and 11βOH-A4 levels decreased, while DHEA-S levels remained unchanged. A4 is the primary precursor of testosterone and A4 produced in the adrenal contributes to testosterone biosynthesis either by secretion or peripheral conversion of the precursor [3]. A reduction in circulating levels of A4 and testosterone by Rooibos may have clinical implications. However, in a study conducted in human test subjects, an increase in testosterone levels after Rooibos consumption for 6 weeks was observed. In addition, DHEA-S levels in men remained unchanged, while a considerable increase in DHEA-S levels were detected in women (results unpublished).

To date the function of 11βOH-A4 in human adrenal steroidogenesis remains uncertain. In a recent study investigating the inhibition of estrogen biosynthesis in gonadal masculinization of rainbow trout, Vizziano et al. [55] showed that masculinization was induced by the inhibition of CYP19 in genetic all-female populations following the administration of 11βOH-A4. The production of this steroid metabolite, exhibiting weak androgenic activity [56] and inhibitory effects on CYP19, which catalyses the biosynthesis of estrone and β-estradiol, could have implications in the output of adrenal steroidogenesis. In this study we found that 11βOH-A4 comprised only 2.3% of the total steroids assayed while A4 comprised 20.5% under basal conditions (Table 1). Upon forskolin stimulation, the production of 11βOH-A4 significantly increased (4.5-fold), comprising 3.5% of the total steroids. Although Xing et al. [3] reported a 5-fold increase in 11βOH-A4 levels upon forskolin stimulation, they found that in adult adrenal cells under basal conditions, A4 and 11βOH-A4 levels comprised 4- and 9% of the total steroids respectively. However, a 17-fold increase was observed in 11βOH-A4 levels following ACTH stimulation. Nevertheless, 11βOH-A4 appears to be stimulated by Rooibos, with a significant increase (1.9-fold) being detected under basal conditions. The production of this metabolite is significantly inhibited by nothofagin (3-fold) only, demonstrating the complex nature of the extract. Following forskolin stimulation, 11βOH-A4 production was inhibited significantly by Rooibos and dihydrochalcone compounds, with a concomitant decrease in A4 levels.

The data shows that while aspalathin and nothofagin are of the most abundant flavonoids in Rooibos [57] and demonstrated similar inhibitory effects, they did not in all cases reflect the effects brought about by Rooibos, clearly indicating that other compounds contribute to the effects of Rooibos on adrenal steroidogenesis. The observation that Rooibos is able to significantly reduce glucocorticoid production during forskolin treatment, but not under basal conditions, may have therapeutic applications for Rooibos in the management of stress-related conditions. In addition, Rooibos resulted in a much greater inhibition (4-fold) in the total amount of steroids detected under forskolin stimulated conditions

Table 3
Steroids produced in forskolin-stimulated H295R cells in the presence of Rooibos and dihydrochalcones. Cells were incubated for 48 h with forskolin (10 μM), Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM). Steroids were quantified by UPLC–MS/MS. Percentage of each steroid ± SEM was calculated by dividing the amount of individual steroid by the total steroid. Fold change ± SEM in response to Rooibos, aspalathin and nothofagin treatment, was calculated from the changes in absolute values of individual steroids (not shown) compared to forskolin values. P values were calculated using a one-way ANOVA with a Dunnett's post test.

Steroid metabolite	Forskolin	Forskolin + Rooibos		Forskolin + aspalathin		Forskolin + nothofagin	
	% Total ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM
PREG	0.7 ± 0.2	8.7 ± 1.1	↑4.2 ± 1.6*	0.6 ± 0.2		1.1 ± 0.1	
PROG	0.0 ± 0.0	0.9 ± 0.1		0.0 ± 0.0		0.1 ± 0.0	
DOC	3.0 ± 0.2	3.7 ± 0.2	↓3.4 ± 0.6**	3.2 ± 0.1		5.8 ± 0.3	
CORT	18.6 ± 0.4	14.1 ± 0.4	↓5.2 ± 0.3***	20.8 ± 0.8		21.6 ± 0.4	
18OH-CORT	0.9 ± 0.0	1.8 ± 0.0	↓2.1 ± 0.3***	1.0 ± 0.0		1.1 ± 0.1	
ALDO	0.2 ± 0.0	0.6 ± 0.0		0.3 ± 0.0		0.4 ± 0.0	
11-DHC	0.1 ± 0.0	0.3 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	
17OH-PREG	ND	ND		ND		ND	
17OH-PROG	0.3 ± 0.0	6.3 ± 0.1	↑4.9 ± 0.2***	0.3 ± 0.0		0.2 ± 0.1	
16OH-PROG	0.6 ± 0.0	0.8 ± 0.1	↓3.1 ± 0.4***	0.7 ± 0.0		0.7 ± 0.0	
Deoxycortisol	24.6 ± 1.0	19.4 ± 0.1	↓5.1 ± 0.6***	23.7 ± 1.8		28.5 ± 0.4	
Cortisol	34.2 ± 1.2	27.8 ± 1.2	↓4.9 ± 0.2***	34.7 ± 1.3	↓1.3 ± 0.1***	28.8 ± 0.6	↓1.7 ± 0.2***
Cortisone	0.1 ± 0.0	0.1 ± 0.0	↓5.2 ± 1.2**	0.1 ± 0.0		0.1 ± 0.0	↓2.6 ± 0.4*
DHEA	ND	ND		ND		ND	
DHEA-S		0.1 ± 0.0					
A4	12.5 ± 0.4	9.3 ± 0.5	↓5.5 ± 0.7***	10.3 ± 0.2	↓1.7 ± 0.2***	9.1 ± 0.3	↓2.0 ± 0.2***
11BOH-A4	3.5 ± 0.3	6.0 ± 0.5	↓2.3 ± 0.0***	3.8 ± 0.2	↓1.3 ± 0.1**	2.1 ± 0.1	↓2.5 ± 0.4***
Testosterone	0.5 ± 0.0	0.1 ± 0.0	↓22.8 ± 5.2***	0.4 ± 0.0	↓1.7 ± 0.2***	0.3 ± 0.0	↓2.4 ± 0.2***
DHT	ND	ND		ND		ND	
Estrone	ND	ND		ND		ND	
β-Estradiol	ND	ND		ND		ND	
Total steroid (nM)	11,141	2788	↓4.0***	8248	↓1.4*	7786	↓1.4*

ND, not detectable ($n = 3$).
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

compared to the 2-fold inhibition observed under basal conditions. A recent study by Beltrán-Debóna et al. [58] showed negligible effects of Rooibos in animals with no metabolic disturbance, while significant reductions in serum cholesterol, triglyceride and free fatty acid concentrations were observed in hyperlipemic mice.

Although the addition of Rooibos led to a reduction in overall biosynthesis of steroid hormones, the data shows that under basal conditions the steroid metabolites in the mineralocorticoid pathway are not significantly affected by the extract or by the two dihydrochalcones, while a decrease in the flux is evident upon forskolin stimulation in the presence of the extract only. While cortisol and cortisone levels are unchanged, the glucocorticoid intermediates are decreased under basal conditions by Rooibos and both compounds. However, upon stimulation with forskolin the inhibitory effect of Rooibos on the flux through the pathway, which results in significant inhibition of cortisol and cortisone production, is greater than that of aspalathin and nothofagin. Similarly, it would appear that a reduction of the flux through the androgen precursor pathway is more pronounced in the case of forskolin stimulation in the presence of the Rooibos extract.

In conclusion, our data indicates that Rooibos and the flavonoid compounds, aspalathin and nothofagin, interact with, and inhibit the steroidogenic enzymes influencing the shunt of metabolites in the mineralocorticoid, glucocorticoid and androgen pathways. The UPLC–MS/MS method developed for this study can be applied in the analysis and accurate quantification of adrenal steroid metabolites and the steroid flux through these precursor pathways. In addition, this method would be applicable in the assessment of inhibition profiles of compounds impacting steroidogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.11.003.

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Supplementary table 1: Gradient specifications of the LC system.

Step	Time (min)	Solvent A (%)	Solvent B (%)	Curve
1	0.00	85	15	1
2	0.04	85	15	6
3	3.12	65	35	5
4	4.67	62	38	6
5	5.45	61	39	6
6	7.00	60	40	7
7	8.50	53	47	6
8	9.50	30	70	6
9	9.55	0	100	6
10	10.00	0	100	6
11	10.10	85	15	6
12	13.00	85	15	6

Supplementary table 2: Parameters for the detection and quantification of 21 steroids by UPLC-MS/MS: retention times (RT, min), cone voltages (CV), collision energy (CE), limit of detection (LOD) and limit of quantification (LOQ).

Steroid metabolite	RT (min)	Precursor ion	CV	Product ion A	CE	Product ion B	CE	Product ion C	CE	LOD[a] (ng/ml)	LOQ[b] (ng/ml)	Calibration range (ng/ml)	Linearity (r^2)
PREG	**9.09**	317.2	16	159.1	18	281.2	12			100	100	100 - 4000	0.995
PROG	**9.11**	315.2	30	96.9	15	297.2	15			0.2	0.2	0.2 - 4000	0.997
DOC	**6.89**	331.2	30	97.0	15	108.9	15			2	2	2 - 4000	0.997
CORT	**4.44**	347.0	30	121.0	15	329.1	15			20	20	20 - 4000	0.998
18OH-CORT	**2.98**	363.2	30	147.0	22	251.2	20	269.2	15	2	2	2 - 4000	0.999
ALDO	**2.90**	361.4	30	97.9	32	315.1	20	343.2	18	0.2	0.2	0.2 - 4000	0.997
11-DHC	**4.09**	345.3	30	121	20	301.2	25			2	2	2 - 4000	0.997
17OH-PREG	**6.81**	297.2	30	165.6	25	256.0	10	297.2	4	200	500	500 - 4000	0.990
17OH-PROG	**7.41**	331.2	30	97.0	15	108.9	15			20	20	20 - 4000	0.998
16OH-PROG	**5.11**	331.2	30	97.0	15	108.9	15			20	20	20 - 4000	0.998
Deoxycortisol	**4.82**	347.0	30	97.9	15	108.9	15			2	2	2 - 4000	0.997
Cortisol	**3.15**	363.0	30	121.0	20					2	2	2 - 4000	0.999
Cortisone	**3.38**	361.2	30	163.0	30					0.2	0.2	0.2 - 4000	0.999
DHEA	**6.63**	271.2	30	243.0	15	253.2	15			100	100	100 - 4000	0.994
DHEA-S	**5.69**	367.2	50	97.0	35					0.2	0.2	0.2 - 4000	0.998
A4	**6.71**	287.2	30	96.9	15	108.8	15			2	2	2 - 4000	0.998
11BOH-A4	**4.07**	303.2	30	121	30	267.2	15			2	20	20 - 4000	0.998
Testosterone	**6.30**	289.2	30	97.2	22	109.0	22			2	2	2 - 4000	0.994
DHT	**7.66**	291.2	25	255.0	15	273.0	20			100	100	100 - 4000	0.994
Estrone	**6.87**	271.2	30	243.0	15	253.2	15			100	100	100 - 4000	0.986
β-Estradiol	**5.74**	255.2	15	133.0	15	159.0	15			100	100	100 - 4000	0.967

[a] Limit of detection was defined as a S/N ratio > 3

[b] Limit of quantification was defined as a S/N ratio > 10

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Article

Rooibos Flavonoids Inhibit the Activity of Key Adrenal Steroidogenic Enzymes, Modulating Steroid Hormone Levels in H295R Cells

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Abstract: Major rooibos flavonoids—dihydrochalcones, aspalathin and nothofagin, flavones—orientin and vitexin, and a flavonol, rutin, were investigated to determine their influence on the activity of adrenal steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase (3βHSD2) and cytochrome P450 (P450) enzymes, P450 17α-hydroxylase/17,20-lyase (CYP17A1), P450 21-hydroxylase (CYP21A2) and P450 11β-hydroxylase (CYP11B1). All the flavonoids inhibited 3βHSD2 and CYP17A1 significantly, while the inhibition of downstream enzymes, CYP21A2 and CYP11B1, was both substrate and flavonoid specific. The dihydrochalcones inhibited the activity of CYP21A2, but not that of CYP11B1. Although rutin, orientin and vitexin inhibited deoxycortisol conversion by CYP11B1 significantly, inhibition of deoxycorticosterone was <20%. These three flavonoids were unable to inhibit CYP21A2, with negligible inhibition of deoxycortisol biosynthesis only. Rooibos inhibited substrate conversion by CYP17A1 and CYP21A2, while the inhibition of other enzyme activities was <20%. In H295R cells, rutin had the greatest inhibitory effect on steroid production upon forskolin stimulation, reducing total steroid output 2.3-fold, while no effect was detected under basal conditions. Nothofagin and vitexin had a greater inhibitory effect on overall steroid production compared to aspalathin and orientin, respectively. The latter compounds contain two hydroxyl groups on the B ring, while nothofagin and vitexin contain a single hydroxyl group. In addition, all of the flavonoids are glycosylated, albeit at different positions—dihydrochalcones at C3' and flavones at C8 on ring A, while rutin, a larger molecule, has a rutinosyl moiety at C3 on ring C. Structural differences regarding the number and position of hydroxyl and glucose moieties as well as

structural flexibility could indicate different mechanisms by which these flavonoids influence the activity of adrenal steroidogenic enzymes.

Keywords: *Aspalathus linearis*; rooibos polyphenols; adrenal steroidogenesis; cytochrome P450 enzymes; UPLC-MS/MS; adrenal H295R cells; cortisol; metabolic disorder; structure-activity relationship; stress

1. Introduction

Flavonoids are a diverse group of plant-derived polyphenols that occur naturally in fruits, vegetables, teas and herbs. These secondary plant metabolites have been shown to exhibit potent anti-oxidant activities, scavenging reactive radicals that cause cellular damage associated with many diseases and clinical conditions. Flavonoids, often referred to as phytoestrogens due to their structural similarity to estrogen and ability to bind the estrogen receptor, may have implications in various clinical conditions and hormone-dependent cancers [1,2]. Although flavonoid polyphenols are generally associated with beneficial health properties, it has been suggested that when consumed in high dosages, flavonoids may act as pro-oxidants and mutagens resulting in cytotoxicity. One such flavonoid, apigenin, a common dietary flavone which exhibits anti-inflammatory, anti-oxidant and anti-carcinogenic properties, was recently shown to induce oxidative stress, causing liver damage in Swiss mice following administration of high dosages of the compound [3,4]. Flavonoids have also been shown to modulate key enzymes in adrenal steroidogenesis, affecting steroid hormone biosynthesis in the mineralocorticoid, glucocorticoid and adrenal androgen pathways [5–8]. Abnormal adrenal steroid hormone levels impact on human health, leading to a broad spectrum of clinical conditions. Chronically elevated cortisol levels have, for example, been associated with metabolic disorders such as visceral obesity, insulin resistance, hypertension, cardiovascular disease and type 2 diabetes [9]. Clinical strategies employed in the treatment of diseases associated with endocrine disorders and cancers include selective inhibitors of either the enzymes catalysing adrenal steroid biosynthesis and metabolism or antagonist activity at steroid receptor level [10]. Diets supplemented with flavonoid-rich plant based foods and beverages may influence the endocrine system and impact on metabolic diseases.

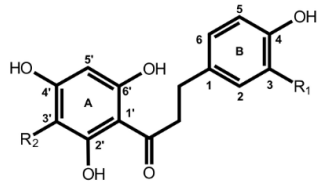
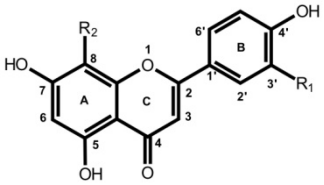
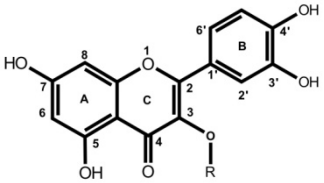
Rooibos, a polyphenol-rich herbal tea prepared as an infusion from the stems and leaves of the plant *Aspalathus linearis*, has traditionally been used to aid in the alleviation of sleeplessness, anxiety and nervous tension, ailments related to stress and physiological conditions generally associated with high cortisol levels. Our previous studies showed that rooibos and specific flavonoids inhibit cytochrome P450 (P450) enzymes and cortisol production [8,11].

In vitro studies in COS-1 cells have shown that unfermented rooibos extract as well as the two rare dihydrochalcones, aspalathin and nothofagin, significantly inhibited P450 17α-hydroxylase/17,20 lyase (CYP17A1) and P450 21-hydroxylase (CYP21A2), while also significantly reducing the levels of cortisol in forskolin stimulated adrenal H295R cells. Even though aspalathin and nothofagin are the two most abundant flavonoids in unfermented rooibos, they did not in all cases reflect the same

inhibitory effect on steroid levels in H295R cells as brought about by the rooibos extract, suggesting that other compounds contribute to the overall effect of rooibos on adrenal steroidogenesis [8].

In the present study we continued our investigation into the influence of rooibos and polyphenolic compounds, belonging to three distinct classes of flavonoids, on adrenal steroidogenic enzymes. The five major rooibos flavonoids used in this study included the dihydrochalcones, aspalathin and nothofagin, their flavone analogues, orientin and vitexin, as well as the flavanol, rutin (Table 1). Due to the structural differences of the flavonoids, these compounds may interact differently with the steroidogenic enzymes thus affecting steroid hormone biosynthesis in the adrenal. We therefore determined the inhibitory effect of rooibos flavonoids on enzymes that play a key role in adrenal steroidogenesis, 3β-hydroxysteroid dehydrogenase (3βHSD2), CYP17A1, CYP21A2 and 11β-hydroxylase (CYP11B1), as well as on overall steroid hormone production in the human adrenal H295R cell line under both basal and forskolin stimulated conditions.

Table 1. Chemical structures of the major rooibos flavonoids.

Structure	Compound	Substitution
Dihydrochalcones 	Aspalathin	R_1=OH, R_2=C-β-D-glucopyranosyl
	Nothofagin	R_1=H, R_2=C-β-D-glucopyranosyl
Flavones 	Orientin	R_1=OH, R_2=C-β-D-glucopyranosyl
	Vitexin	R_1=H, R_2=C-β-D-glucopyranosyl
Flavonol 	Rutin	R=rutinosyl

2. Results and Discussion

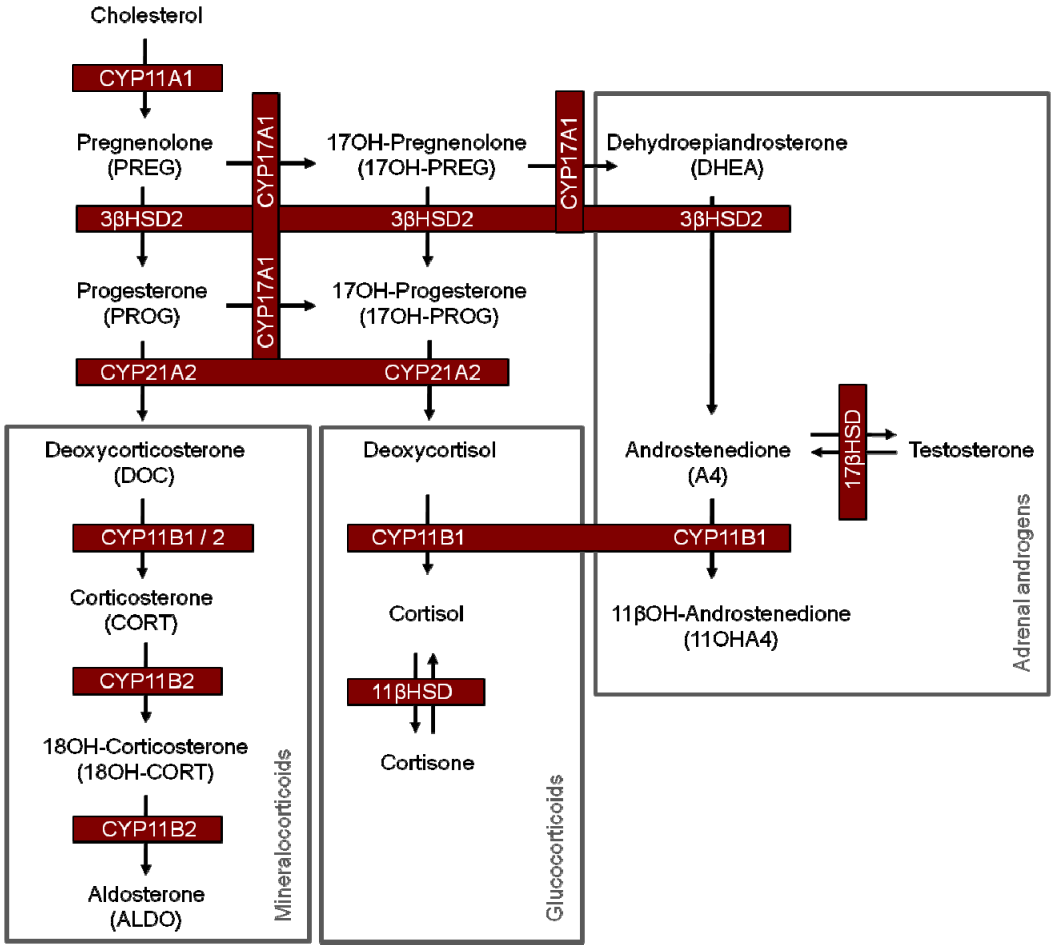
It has been widely reported that polyphenols exhibit a diverse range of beneficial biological effects, and as such, their consumption as part of the daily diet cannot be ignored. Although teas and herbal infusions contribute to the daily intake of dietary flavonoids, the pharmacological actions of these extracts cannot directly be attributed to single compounds within these beverages, unless identified and assayed in isolation. Flavonoids modulate steroid hormone biosynthesis and metabolism due to their interaction with steroidogenic enzymes, either inhibiting or stimulating specific enzymes [7,8]. We recently showed that unfermented rooibos extract influenced the steroid flux in the mineralocorticoid, glucocorticoid and androgen precursor pathways in human adrenal H295R cells under both basal and

forskolin stimulated conditions [8]. While the effect of flavonoid compounds on the flux through the adrenal steroidogenic pathways can be determined, the influence on specific enzymes in these pathways is less obvious due to upstream inhibition of steroid intermediates. In the present study, we assayed the effect of flavonoid compounds on individual steroidogenic enzymes as well as on steroid metabolite levels in H295R cells using ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS).

2.1. The Influence of Rooibos and Selected Flavonoid Compounds on Adrenal Steroidogenic Enzymes Expressed in Non-Steroidogenic COS-1 Cells

CYP17A1 plays a central role at the branch point in adrenal steroid biosynthesis, and together with 3βHSD2, determines the shunt of steroid metabolites in the glucocorticoid, mineralocorticoid and adrenal androgen pathways (Scheme 1).

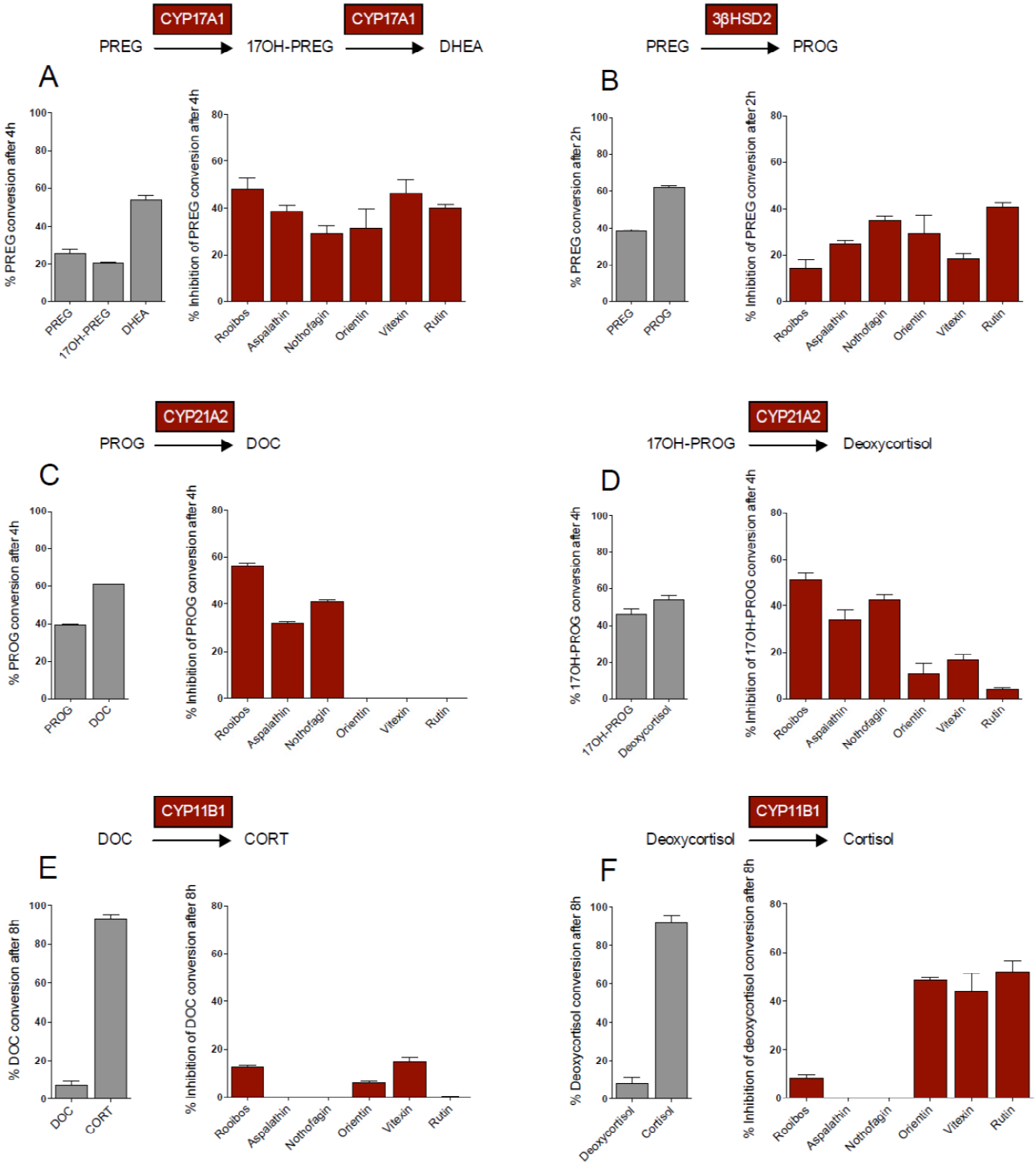
Scheme 1. Steroid hormones produced in human adrenal cells.



CYP17A1 catalyses the 17α-hydroxylation of pregnenolone (PREG), yielding 17OH-pregnenolone (17OH-PREG), which is in turn converted to dehydroepiandrosterone (DHEA). These three metabolites are substrates for 3βHSD2, which catalyzes their conversion to progesterone (PROG), 17OH-progesterone (17OH-PROG), and androstenedione (A4), respectively, thereby shunting metabolites into the respective pathways. In addition, CYP17A1 also catalyses the conversion of PROG to

17OH-PROG and 16OH-progesterone (16OH-PROG), with the latter not being further metabolized in the adrenal. PREG conversion by CYP17A1 was inhibited by all the flavonoid compounds assayed, with the compounds exhibiting similar inhibitory effects (Figure 1A). Although PREG conversion by 3βHSD2 was also inhibited by all the flavonoids assayed, rutin exhibited the greatest inhibitory effect, while vitexin had the lowest inhibitory effect, 2-fold lower than that of rutin (Figure 1B).

Figure 1. Substrate conversion (1 μM) in transiently transfected COS-1 cells in the absence and presence of rooibos extract (4.3 mg/mL) and selected flavonoids (10 μM). CYP17A1 and CYP21A2 data adapted from [8].



CYP21A2 catalyzes the conversion of PROG and 17OH-PROG to DOC and deoxycortisol, respectively. In contrast to CYP17A1 and 3βHSD2, only the dihydrochalcones inhibited the activity of CYP21A2 significantly, with the flavones and rutin not influencing the conversion of PROG by CYP21A2 (Figure 1C). In the conversion of 17OH-PROG, however, these compounds displayed inhibitory effects below 20% (Figure 1D). Neither aspalathin nor nothofagin inhibited the conversion of DOC and deoxycortisol by CYP11B1 to their corresponding products, CORT and cortisol, respectively. The main structural difference between the dihydrochalcones and the other flavonoids assayed in this study is the structural flexibility of the molecules and the glucose moiety at C3' on ring A of the dihydrochalcones, which could interfere with the binding of the compounds to CYP11B1. Inhibition of CYP11B1 by vitexin, orientin and rutin, however, was substrate specific. While inhibition of DOC conversion was negligible (Figure 1E), inhibition of deoxycortisol conversion was significantly higher in the presence of rutin, orientin and vitexin (Figure 1F). The higher binding affinity of CYP11B1 for DOC compared to deoxycortisol may account for the negligible inhibition of the flavonoids on the conversion of DOC. CYP11B1 has been shown to exhibit a lower Km for DOC and its catalytic conversion is also characterized by a lower K_{cat}, 2-fold less than that of deoxycortisol conversion, clearly indicating that a single hydroxyl group influences the enzyme's affinity for its substrate, as well as the substrate turnover. It was suggested that the presence of the C17 hydroxyl group of deoxycortisol obstructs the entrance of deoxycortisol into the enzyme's active pocket [12].

While the rooibos extract exhibited the greatest inhibitory effect on CYP17A1 and CYP21A2, inhibition of the other enzymes was negligible. The inhibition of PREG conversion by CYP17A1 (Figure 1A) as well as the conversion of PROG (Figure 1C) and 17OH-PROG (Figure 1D) by CYP21A2 was similar (±50%) in the presence of the extract. However, the inhibitory effect of rooibos on PREG conversion by 3βHSD2 (Figure 1B) and DOC (Figure 1E) and deoxycortisol (Figure 1F) conversion by CYP11B1, was less than 20%. The inhibitory effects brought about by the individual flavonoid compounds (10 μM) was not reflected in the inhibitory effects of the whole extract in all instances, even though the flavonoid concentrations within the rooibos extract ranged between 27 μM and 1.4 mM. Although we assayed five of the major flavonoids present in rooibos, it is important to note that, to date, 46 flavonoid compounds have been identified in rooibos [13,14] which may possibly contribute to the data obtained in the presence of the extract. From the data it is clear, however, that the rooibos extract preferentially inhibits CYP17A1 and CYP21A2, two key enzymes at the branch point of adrenal steroidogenesis.

2.2. The Influence of Selected Flavonoids on Steroid Metabolism in Adrenal H295R Cells under Basal and Forskolin Stimulated Conditions

In H295R cells, the dihydrochalcones and the flavones decreased the total steroid output under both basal and stimulated conditions, while rutin's effect was only detected in the presence of forskolin. Forskolin mimics the effects of ACTH by activating cAMP pathways in adrenal cells [15], stimulating steroidogenic enzymes and increasing steroid production. As expected, forskolin significantly increased the total steroid output to 11.1 μM (2.8 fold), with the greatest inhibition being detected in the presence of rutin (Table 2). Both aspalathin and nothofagin significantly reduced basal

17OH-PROG and 16OH-PROG levels due to the inhibition of CYP17A1 and 3βHSD2. Nothofagin also reduced deoxycortisol levels under basal conditions, possibly due to inhibition of CYP21A2 or due to lower levels of 17OH-PROG being available as the precursor substrate. Both dihydrochalcones reduced A4 levels significantly under basal and forskolin stimulated conditions, indicative of 3βHSD2 inhibition. Interestingly, while nothofagin reduced basal levels of 11β-hydroxyandrostenedione (11OHA4), the product of A4, it did not reduce basal production of cortisol, which is catalysed by the same enzyme, CYP11B1. Although this may suggest possible inhibition of CYP11B1, neither aspalathin nor nothofagin were shown to inhibit the enzyme at 10 μM in COS-1 cells, suggesting that the reduction in 11OHA4 was due to upstream inhibition. Under stimulated conditions, the production of both cortisol and 11OHA4 was inhibited in the presence of aspalathin and nothofagin, indicating upstream inhibition, resulting in reduced levels of precursor steroid metabolites. It should, however, also be noted that when comparing inhibitory effects of the flavonoid compounds under basal and stimulated conditions, the expression levels of steroidogenic enzymes are altered when cells are stimulated with forskolin, which would in turn alter steroid metabolite/precursor levels. From these results it is clear that the inhibitory effects of both dihydrochalcones are very similar, however, the inhibitory effect of nothofagin was notably greater than that of aspalathin.

Table 2. Steroid metabolites produced in adrenal H295R cells under basal and forskolin (10 μM) stimulated conditions in the absence and presence of selected flavonoids (10 μM) after 48 h.

Steroid	**Basal**	**+ Aspalathin [a]**	**+ Nothofagin [a]**	**+ Orientin**	**+ Vitexin**	**+ Rutin [b]**
metabolites	**Total ± SEM (nM)**	**Fold change**	**Fold change**	**Fold change**	**Fold change**	**Fold change**
PREG	35.2 ± 2.2				↑ 1.4 **	
PROG	5.8 ± 1.7					
DOC	107.4 ± 13.0			↑ 1.2 *	↑ 1.2 **	
CORT	241.1 ± 31.6			↑ 1.2 *		
18OH-CORT	9.3 ± 1.3			↑ 2.6 *		
ALDO	2.3 ± 0.5					
11-DHC	9.9 ± 0.3					
16OH-PROG	66.4 ± 10.3	↓ 2.3 ***	↓ 3.6 ***	↓ 1.2 ***	↓ 1.8 ***	
17OH-PROG	56.8 ± 6.3	↓ 3.6 **	↓ 4.8 **		↓ 1.4 *	
Deoxycortisol	1741.0 ± 234.1		↓ 2.3 *		↓ 1.3 ***	
Cortisol	670.2 ± 39.3				↓ 1.3 **	
Cortisone	6.6 ± 2.2			↓ 1.5 ***	↓ 1.8 ***	
A4	806.5 ± 115.6	↓ 2.4 **	↓ 3.5 ***	↓ 1.1 *	↓ 1.5 ***	
11OHA4	90.1 ± 11.1		↓ 3.0 *		↓ 1.5 ***	
Testosterone	39.22 ± 3.60	↓ 4.3 ***	↓ 7.1 ***	↓ 1.3 **	↓ 2.5 ***	
Total steroid (nM)	3947.0	↓ 1.6	↓ 2.0 *	↓ 1.0	↓ 1.3 **	↓ 1.0

Table 2. *Cont.*

Steroid metabolites	Forskolin Total ± SEM (nM)	+ Aspalathin [a] Fold change	+ Nothofagin [a] Fold change	+ Orientin Fold change	+ Vitexin Fold change	+ Rutin [b] Fold change
PREG	47.5 ± 3.4			↑ 1.4 **	↑ 1.3 **	
PROG	5.1 ± 0.5			↑ 1.4 **	↑ 1.5 **	
DOC	343.2 ± 61.1			↓ 1.2 *	↓ 1.1 *	
CORT	2062.0 ± 170.5				↓ 1.3 **	↓ 1.9 *
18OH-CORT	104.8 ± 15.1					↓ 2.0 **
ALDO	27.4 ± 3.4			↓ 1.1 ***	↓ 1.4 *	
11-DHC	11.2 ± 1.4					
16OH-PROG	66.0 ± 4.7				↓ 1.4 **	
17OH-PROG	36.0 ± 2.4					↓ 3.1 *
Deoxycortisol	2757.0 ± 393.1				↓ 1.3 *	↓ 2.0 **
Cortisol	3793.0 ± 285.1	↓ 1.3 ***	↓ 1.7 ***	↓ 1.3 ***	↓ 1.8 ***	↓ 3.0 ***
Cortisone	11.6 ± 2.7		↓ 2.6 *			
A4	1402.0 ± 180.0	↓ 1.7 ***	↓ 2.0 ***	↓ 1.4 ***	↓ 1.5 ***	↓ 4.4 ***
11OHA4	388.3 ± 16.3	↓ 1.3 **	↓ 2.5 ***	↓ 1.5 ***	↓ 1.7 ***	↓ 6.9 ***
Testosterone	50.5 ± 6.1	↓ 1.7 ***	↓ 2.4 ***	↓ 1.7 ***	↓ 2.3 ***	↓ 5.8 ***
Total steroid (nM)	11,141.0	↓ 1.4 *	↓ 1.4 *	↓ 1.1	↓ 1.3 **	↓ 2.3 **

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; [a] Adapted from [8]; [b] Adapted from [11]; ↑ (increase) or ↓ (decrease) in steroid levels (nM) under basal / forskolin stimulated conditions in the presence of flavonoids.

Orientin, the flavone analogue of aspalathin, significantly increased the steroid flux in the mineralocorticoid pathway, increasing basal levels of DOC, CORT and 18OH-CORT, suggesting that inhibition of CYP17A1 resulted in more metabolites being channeled into the mineralocorticoid pathway. The data obtained clearly shows that the flavones inhibit CYP17A1, since PREG and PROG levels increased, while 16OH-PROG and 17OH-PROG levels decreased (Table 2).

The inhibitory profile of vitexin was very similar to that of orientin, however, vitexin exhibited a greater inhibitory effect, reducing the total steroid output 1.3-fold under both basal and stimulated conditions, while the effect of orientin on total steroid production was negligible. Interestingly, under stimulated conditions, during which aldosterone synthase (CYP11B2) expression is upregulated [16], the effect of vitexin on the mineralocorticoid pathway was much more prominent compared to orientin, with a greater reduction in ALDO levels being observed, suggesting inhibition of either CYP11B2 or upstream enzymes. Under both basal and stimulated conditions, vitexin also had a much greater effect on the glucocorticoid pathway compared to orientin, reducing the levels of cortisol and its precursors significantly. In COS-1 cells, rutin as well as the flavones inhibited the biosynthesis of cortisol by CYP11B1, while the dihydrochalcones did not, suggesting that the reduced cortisol levels observed in H295R cells in the presence of aspalathin and nothofagin may be indicative of upstream inhibition only.

While the dihydrochalcones and their flavone analogues reduced basal testosterone levels significantly, it was the former compounds which had the greatest effect, suggesting inhibition of 17βHSD. However, the adrenal is not the primary site for 17βHSD expression, as is also the case for 11βHSD2, which catalyses the formation of cortisone and 11-dehydrocorticosterone (11-DHC). The detected levels of these metabolites were very low, with the flavones reducing cortisone levels

under basal conditions, while under stimulated conditions, it was inhibited in the presence of nothofagin only. Although 17βHSD and 11βHSD2 are expressed at very low levels in the adrenal [17], it would appear that these hydroxysteroid dehydrogenases, together with 3βHSD2, are sensitive to the flavonoid compounds present in Rooibos.

In a recent pharmacophore-based virtual screening study investigating inhibitors of 17βHSD type 3 and 5, key enzymes involved in adrenal androgen production, it was shown that the ligand binding domain of these enzymes is able to accommodate structurally highly diverse ligands which bind to different regions of the active site. An enlargement of the binding cavity was observed in the crystal structure of 17βHSD3/5 when rutin was bound to these enzymes. Furthermore, the X-ray structure of rutin bound to 17βHSD5 showed that water molecules formed a hydrogen bonding network with rutin bound at the base of the ligand binding domain [18].

Rutin, one of the more stable flavonoid compounds in both fermented and unfermented Rooibos, had no significant effect on any of the steroid hormones under basal conditions. However, upon forskolin stimulation, rutin had the greatest inhibitory effect of all the flavonoid compounds assayed, reducing the total steroid output 2.3-fold. In the mineralocorticoid pathway, CORT and 18OH-CORT levels were significantly lower, suggesting inhibition of CYP11B2. In the glucocorticoid pathway, significant reductions in the levels of 17OH-PROG, deoxycortisol and cortisol indicate inhibition of CYP17A1 and CYP11B1, as was also shown in COS-1 cells (Figure 1). Upstream inhibition also contributed to the reduced deoxycortisol levels, since rutin did not inhibit CYP21A2. In the adrenal androgen precursor pathway, rutin reduced the levels of A4, testosterone and 11OHA4, suggesting inhibition of CYP17A1 and/or 3βHSD2.

The data obtained in the H295R cell line did not in all cases reflect results obtained in COS-1 cells. The expression levels of the individual enzymes assayed in COS-1 cells are comparable, as the cells were transiently transfected with equal concentrations of the appropriate cDNA. Furthermore, the conversion of substrates in COS-1 cells allowed assays to be conducted under very specific conditions. A fixed substrate concentration and no competition from other enzymes for the same substrates, as is the case in the adrenal H295R cell model, allowed us to investigate the effect of flavonoids on the catalytic activity of specific steroidogenic enzymes. In contrast, enzymes are expressed at different levels in adrenal H295R cells and vary from enzyme to enzyme under both basal and forskolin stimulated conditions, with specific enzymes being upregulated when cells are stimulated. It was recently shown that under basal conditions in H295R cells, CYP11A1 is expressed at the highest levels, followed by CYP21A2, CYP17A1, SULT2A1, 3βHSD2, CYP11B2 and CYP11B1, while under forskolin stimulated conditions, CYP17A1 is expressed at the highest levels, followed by CYP21A2, CYP11A1, 3βHSD2, SULT2A1, CYP11B2, and CYP11B1 [16].

Overall, from these results it is clear that orientin and vitexin had the greatest effect on the mineralocorticoid pathway under both basal and forskolin stimulated conditions. The dihydrochalcones had no significant effect on any of the metabolites in the mineralocorticoid pathway, while rutin's effect was only detected under stimulated conditions, with significantly reduced levels of ALDO precursors, CORT and 18OH-CORT. The influence of rutin was most evident in the glucocorticoid and adrenal androgen pathways under stimulated conditions, with the other flavonoids decreasing metabolites under both basal and stimulated conditions in these pathways.

2.3. Structure Activity Analyses

Analyses regarding the influence of structural differences of flavonoid compounds on their ability to inhibit steroidogenic enzymes are hampered due to the uncertainty of the manner in which these compounds would interact with the active site of these enzymes. As the type of inhibition of the steroidogenic cytochromes P450 by the flavonoids is not known, it is possible that binding could also occur at sites other than the active pocket. Furthermore, molecular docking studies reporting on structural aspects of flavonoid compounds impacting on substrate inhibition in terms of steroidogenic enzymes are limited. It has, however, been shown by Ohno *et al.* that the isoflavone, diadzein, was able to competitively inhibit the binding of DHEA and PROG to 3βHSD2 and CYP21A2, respectively. The authors suggested, having assayed a range of flavone compounds, that it was the hydroxyl groups at C6 on ring A and C4' on ring B which played an important role in the inhibition of steroidogenic P450 enzymes. They showed, in cAMP-stimulated H295R cells, at a concentration of 12.5 μM, that 6-hydroxyflavone, 4'-hydroxyflavone and apigenin, which contains hydroxyl groups at C5, C7 and C4', inhibited cortisol production significantly [7]. In the present study, vitexin, with a C4' hydroxyl group and orientin with C3' and C4' hydroxyl groups, both also containing a glucose moiety at C8, significantly inhibited cortisol biosynthesis at a concentration of 10 μM. In COS-1 cells, it was the flavones and rutin that inhibited CYP11B1 significantly, while the dihydrochalcones did not inhibit the activity of CYP11B1 towards DOC or deoxycortisol. It is interesting to note that the flavones as well as rutin, containing glucose moieties on ring A and ring C respectively, showed negligible inhibition of CYP21A2. In contrast, while aspalathin and nothofagin significantly inhibited substrate conversion by CYP21A2, these compounds had no influence on the conversion of either DOC or deoxycortisol by CYP11B1, possibly due to the glucosyl moiety at C3' on ring A. Our data thus far suggests that a glucosyl moiety at this position may prevent compounds inhibiting the catalytic activity of CYP11B1, while the C8 glucosyl and C3 rutinosyl moieties on the pyran ring of the other flavonoids do not. Substitutions at C3 and C4 (dihydrochalcones) and C3' and C4' (flavones and flavonol) on the B ring may, however, also play a role. The type of inhibition that these compounds elicit need to be determined, while homology models may pinpoint the effect of functional groups on the binding of the compounds in the active pocket of the relevant enzymes.

In a more recent study by Hasegawa *et al.* [5], it was also shown in H295R cells that a range of flavones with hydroxyl groups at positions C4' or C3' and C4' on ring B significantly reduced the levels of DOC and A4, suggesting inhibition of 3βHSD2, while apigenin was more potent than the other polyphenols assayed, increasing the levels of PREG and 17OH-PROG, suggesting inhibition of CYP17A1, CYP21A2 and 3βHSD2. Apigenin was also shown to downregulate the expression levels of CYP17A1, CYP21A2 and 3βHSD2 mRNA significantly [5].

From our data it appears that the enzymes 3βHSD2 and CYP17A1 are most susceptible to inhibition by the rooibos flavonoids assayed in this study. The natural substrates for these two enzymes, PREG, 17OH-PREG and DHEA contains a hydroxyl group on C3 of the steroid backbone structure involved with substrate binding in the active pocket [19,20]. It is possible that the hydroxyl group on position C4' of the flavonoid is involved in the binding of these compounds to the active pocket of steroidogenic P450 enzymes, with hydrogen bonds stabilizing the orientation of flavonoid compounds in the active site. However, since limited data is available on the molecular docking of flavonoids, no

conclusions can be drawn regarding the orientation of these compounds within the active pocket. In addition, as the mechanism of inhibition is uncertain, the binding of these compounds to the same site that the substrate would occupy cannot be assumed. A recent study by Androutsopoulos *et al.* [21], investigating the binding mode of selected flavonoids to the heme group using the CYP1A2 crystal structure as a template, predicted that flavonoids bind in the active pocket with the B-ring orientated towards the heme group. In an earlier study, Shimada *et al.* [22] investigated the inhibition of a range flavonoid compounds towards five human P450 enzymes by assessing inhibitory activity together with molecular docking studies. These studies clearly showed that the position and number of hydroxy and methoxy groups impacted on the orientation of the compounds in the active pocket of the different enzymes. It was shown that ring B of the compounds was not, in all cases, oriented towards the heme group and that hydroxy/methoxy substitutions influenced positioning of the flavonoid compounds and thus also affected the mechanisms of inhibition for the enzymes assayed.

The compounds assayed in this study all contained glucose moieties on either ring A or C, which would, if bound in the active site of the enzyme, affect the orientation of the molecule, impacting on the mechanism of inhibition. It would seem that while a hydroxy group on B ring plays a role in flavonoid inhibitory activity, a second hydroxy group does not significantly affect inhibition or contribute towards increasing inhibition, possibly due to rotation between rings A and B resulting in a degree of flexibility. It should, however, also be noted that aspalathin and nothofagin have a more flexible structure compared to rutin, orientin and vitexin. It is interesting to note that the dihydrochalcones inhibit the catalytic activity of CYP21A2 towards both substrates, while exhibiting no inhibitory effect on CYP11B1, regardless of the substrate. In contrast, the inhibition of the activity of these enzymes towards their substrates differs in the presence of flavone/flavonol compounds, with the inhibitory effect of these compounds being greater when the substrate is hydroxylated at the C17 position.

3. Experimental

3.1. Reagents and Instruments

Unfermented rooibos plant material was provided by the South African Rooibos Council (Rooibos LTD-BPK, Clanwilliam, South Africa). Aspalathin and nothofagin were obtained from Prof. W.C.A. Gelderblom (Medical Research Council, Western Cape, South Africa). Orientin and vitexin were purchased from Extrasynthese (Genay Cedex, France). Rutin, steroid metabolites, forskolin, Dulbecco's modified Eagle's Medium (DMEM) and an MTT assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nucleobond® AX plasmid preparation kits were purchased from Machery-Nagel (Duren, Germany). Mirus TransIT®-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Penicillin–streptomycin, fetal calf serum and trypsin-EDTA were obtained from Gibco BRL (Gaithersburg, MD, USA). Deuterated cortisol (9,11,12,12-D4-cortisol) was purchased from Cambridge isotopes (Andover, MA, USA). DMEM/F_{12} and gentamicin were purchased from Invitrogen/Gibco (Grand Island, New York, USA). Cosmic calf serum was supplied by HyClone®, Thermo Scientific (South Logan, Utah, USA). A bicinchoninic acid (BCA) protein determination kit was purchased from Pierce (Rockford, IL, USA). The UPLC BEH C18 column was purchased from Waters (Milford, MA, USA). The Kinetex PFP column was obtained from

Phenomenex (Torrance, CA, USA). All chemicals were of the highest quality and supplied by reputable scientific suppliers.

3.2. Substrate Conversion Assays in Transiently Transfected COS-1 Cells

COS-1 cells were grown at 37 °C and 5% CO_2 in DMEM containing 0.9 g/L glucose, 0.12% $NaHCO_3$, 10% fetal calf serum and 1% penicillin streptomycin. The cells were plated into 12 well dishes (1×10^5 cells/mL, 1 mL/well), 24 h prior to transfection. Cells were transiently transfected with 0.5 µg cDNA (baboon CYP17A1/pCIneo, baboon 3βHSD2/pCIneo, baboon CYP21A2/pCIneo) and 1.5 µL *Mirus TransIT*®- LT1 transfection reagent according to the manufacturer's instructions. Co-transfections with baboon CYP11B1/pTarget and human ADX/pCIneo cDNA were carried out using 0.25 µg of each plasmid. Control transfection reactions were performed using the pCIneo vector containing no cDNA insert. Cells were incubated for 72 h prior to substrate addition. Substrate conversion (1 µM) was assayed in the absence and presence of aspalathin, nothofagin, rutin, orientin and vitexin, assayed at 10 µM, and unfermented rooibos extract, assayed at 4.3 mg/mL. The methanolic extract of unfermented rooibos plant material used in this study was prepared and analysed as previously described [8,11]. At specific time intervals, 500 µL aliquots were removed and the steroids were extracted using a 10:1 volume of dichloromethane to culture medium. The mixture was vortexed for 30 s, centrifuged at 500 ×*g* for 5 min and the medium removed via aspiration. The dichloromethane phase, containing the steroid metabolites, was dried under N_2, resuspended in 150 µL methanol and stored at −20 °C prior to analyses.

3.3. Steroid Metabolism in Adrenal H295R Cells

H295R cells were grown at 37 °C and 5% CO_2 in DMEM/F_{12}, supplemented with L-glutamine, 15 mM HEPES, pyridoxine, 1.125 g NaHCO3/l, 1% penicillin streptomycin, 0.01% gentamicin, and 10% cosmic calf serum (growth medium). The cells were plated into 12 well dishes (4×10^5 cells/mL, 1 mL/well) and incubated for 48 h. The medium was subsequently replaced with experimental medium (growth medium containing 0.1% cosmic calf serum) and cells were incubated for a further 12 h prior to substrate addition. Steroid metabolism was assayed in the presence of selected flavonoid compounds, aspalathin, nothofagin, rutin, orientin and vitexin (10 µM), under both basal and forskolin (10 µM) stimulated conditions. After 48 h, the medium (500 µL) was removed and 15 ng D4-cortisol added as an internal standard. The steroids were extracted as described in Section 3.2.

3.4. Cell Viability

COS-1 cells were plated out in a 96 well plate (100 µL, 1×10^5 cells/mL) and incubated with selected flavonoid compounds (10 µM) for 8 h. H295R cells were plated out in a 96 well plate (100 µL, 4×10^5 cells/mL) and incubated with selected flavonoids (10 µM) and forskolin (10 µM) for 48 h. Cell viability was assayed using an MTT toxicology assay kit according to the manufacturer's instructions. Color-standardized controls (media containing either 1 mg/mL or 4.3 mg/mL Rooibos extract) were included to compensate for the possible interference of the Rooibos color with the assay. None of the abovementioned test compounds had a significant effect on COS-1 or H295R cell viability.

3.5. Separation and Quantification of Steroid Metabolites Using UPLC-MS/MS

All steroid metabolites were analysed and quantified using UPLC–MS/MS (ACQUITY UPLC, Waters, Milford, MA, USA). Steroid metabolites from conversion assays in COS-1 cells were separated using a Waters UPLC BEH C18 (2.1 mm × 50 mm, 1.7 µm) column, while steroid metabolites produced in H295R cells were separated using a Phenomenex UPLC Kinetex PFP (2.1 mm × 100 mm, 2.6 µm) column, as previously described [11]. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray in the positive ionization mode (ESI+). Gradients of the LC system and other relevant information including the mobile phases, flow rates, injection volumes, parent and daughter ions, cone (V) and collision (eV) voltages, retention times and the validation of the UPLC–MS/MS assays has been described previously [11]. Calibration curves were constructed by using weighted ($1/x^2$) linear least squares regression. Data was collected with the MassLynx 4.0 software program. The data obtained in assays conducted COS-1 cells are depicted as % inhibition of substrate conversion, while the data obtained in H295R cells are expressed as absolute values (nM) ± SEM.

3.6. Statistical Analysis

All results are representative of two independent experiments, performed in triplicate, with the data being expressed as the mean ± SEM. After each assay a protein determination by the Pierce BCA method was performed in order to normalize steroid levels to protein concentrations. Statistical analyses were calculated with GraphPad Prism (version 5) software (GraphPad Software, San Diego, CA, USA) using a one-way ANOVA, followed by a Dunnett's multiple comparison test. A value of $p < 0.05$ was considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. Conclusions

This study included five of the major flavonoid compounds present in rooibos. These flavonoids, regardless of structural differences, all inhibited the activitiy of CYP17A1 and 3βHSD2, branch point enzymes in adrenal steroidogenesis. The dihydrochalcones and the flavone/flavonol compounds, however, showed marked differences in their inhibition of the activity of downstream enzymes, CYP21A2 and CYP11B1, expressed in COS-1 cells. In addition, these compounds also exhibited distinct differences in terms of specific substrates. It is clear that structural differences regarding the number and position of hydroxyl and glucose moieties, and structural flexibility, impact on the inhibitory effect of these flavonoids, possibly indicating different mechanisms. Rooibos extract, which contains a wide spectrum of flavonoid compounds, some of which are present at higher levels than was assayed, did not in all cases exhibit the same degree of inhibition. It is thus plausible that, of the array of compounds present in rooibos, some may inhibit while others may stimulate or have no effect at all. Overall, it is clear that, although the mechanism of inhibition is uncertain, the major rooibos flavonoid compounds assayed in this study significantly influence adrenal steroidogenic enzyme activities and steroid hormone levels.

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Author Contributions

Both authors contributed equally towards the preparation of this manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the rooibos extracts are available from the authors.

RESEARCH ARTICLE

Rooibos influences glucocorticoid levels and steroid ratios in vivo and in vitro: A natural approach in the management of stress and metabolic disorders?

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Scope: To determine the effect of Rooibos (*Aspalathus linearis*) on glucocorticoid biosynthesis and inactivation in vivo and in vitro.

Methods and results: Ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) analyses of in vivo studies showed that human Rooibos consumption increased cortisone plasma levels in males ($p = 0.0465$) and reduced cortisol:cortisone ratios in males and females ($p = 0.0486$) at risk for cardiovascular disease. In rats, corticosterone (CORT) ($p = 0.0275$) and deoxycorticosterone ($p = 0.0298$) levels as well as the CORT:testosterone ratio ($p = 0.0009$) decreased following Rooibos consumption. The inactivation of cortisol was investigated in vitro by expressing 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and type 2 (11βHSD2) in CHO-K1 cells. Rooibos inhibited 11βHSD1, which resulted in a significant reduction in the cortisol:cortisone ratio ($p < 0.01$). No significant effect was detected on 11βHSD2. In vitro studies in adrenal H295R cells showed that Rooibos and rutin, one of the more stable flavonoid compounds present in Rooibos, significantly reduced the levels of cortisol and CORT in cells stimulated with forskolin to mimic a stress response.

Conclusion: In vivo studies demonstrate that Rooibos significantly decreased glucocorticoid levels in rats and steroid metabolite ratios linked to metabolic disorders—cortisol:cortisone in humans and CORT:testosterone in rats. Results obtained at cellular level elucidate possible mechanisms by which these effects were achieved.

Keywords:
Adrenal H295R cells / Cytochrome P450 / Functional food / Metabolic syndrome / Rooibos tea polyphenol flavonoids

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1 Introduction

Rooibos is a popular tisane or herbal tea made from the stems and leaves of the fynbos plant, *Aspalathus linearis*, which is unique to the Western Cape region of South Africa. Rooibos has gained popularity globally as a health drink, and while Germany and the Netherlands have for many years commanded the greatest export market, export of Rooibos to the UK, USA, and Japan has risen steadily, Website:

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Abbreviations: **11βHSD**, 11β-hydroxysteroid dehydrogenase; **11βOHA4**, 11β-hydroxyandrostenedione; **11-DHC**, 11-dehydrocorticosterone; **3βHSD**, 3β-hydroxysteroid dehydrogenase; **CHO-K1**, chinese hamster ovary; **CORT**, corticosterone; **CYP11B1**, cytochrome P450 11β-hydroxylase; **CYP17A1**, cytochrome P450 17α-hydroxylase/17,20-lyase; **CYP21A2**, cytochrome P450 21-hydroxylase; **DHEA-S**, dehydroepiandrosterone-sulfate; **DOC**, deoxycorticosterone; **H6PDH**, hexose-6-phosphate dehydrogenase; **UPLC-MS/MS**, ultra-performance liquid chromatography/tandem mass spectrometry

www.ppecb.com [1, 2]. Although local Rooibos sales have also increased [2], it is consumed mainly as an herbal "tea," competing with coffee and varieties of *Camellia sinensis*. In 1997, functional teas already comprised 25% of tea sales in the USA [3] and Rooibos has since joined the healthy dietary trends as a functional beverage in the USA, (Website: www.PreparedFoods.com) [4]. While Rooibos is caffeine free with very low tannin levels, it is also a rich source of polyphenols. Within this group of compounds, flavonoids have been shown to exhibit a wide range of activities, with the daily consumption of tea contributing substantially toward dietary flavonoid intake. Rooibos contains a unique flavonoid profile, which is altered due to oxidation during fermentation, with the process resulting in a significant loss of antioxidant activity [5]. Rooibos is manufactured both as a fermented and unfermented product. The former is produced by first fermenting shredded plant material for 12–14 h in mounds in which the temperature increases to ± 40°C. This process results in the leaves becoming reddish brown in color, after which the material is sun-dried. Unfermented Rooibos is generally produced by spreading shredded plant material in thin layers to minimize drying time in the sun, thus maintaining the green color and reducing oxidative changes. While fermentation decreases antioxidant activity, aspalathin and nothofagin are the two compounds most affected by the fermentation process [6].

Anecdotal evidence suggests that Rooibos aids in the alleviation of depression, anxiety and insomnia—ailments associated with stress and which are generally linked to the endocrine system. To date, very little clinical data are available on the biological effects of Rooibos on the endocrine system. Although soy flavonoids have been shown to inhibit cortisol production in H295R cells, an adrenal cell model, the effect of phytochemicals on steroidogenesis in vivo remained undetermined [7]. Our recent report showed that Rooibos significantly influenced the outcome of steroid hormone biosynthesis in H295R cells stimulated with forskolin to mimic a stress response [8].

In the adrenal, the biosynthesis of glucocorticoids, mineralocorticoids, and adrenal androgens from the common precursor, cholesterol, is catalyzed by the cytochrome P450 (P450) enzymes and 3β-hydroxysteroid dehydrogenases (3βHSD), with cytochrome P450 11β-hydroxylase (CYP11B1) catalyzing the production of corticosterone (CORT) and cortisol from their respective precursors, deoxycorticosterone (DOC) and deoxycortisol. CORT can also be further metabolized to aldosterone by aldosterone-synthase in the mineralocorticoid pathway [9]. Glucocorticoids mediate their effects via the glucocorticoid receptor, with their availability being dependent on the activity of the 11β-hydroxysteroid dehydrogenase (11βHSD) isozymes. The conversion of cortisol and CORT to their inactive keto-metabolites, cortisone and 11-dehydrocorticosterone (11-DHC), respectively, is catalyzed by 11βHSD type 2 (11βHSD2) (Fig. 1). This enzyme is expressed mainly in mineralocorticoid target tissues such as the kidney and colon, where it protects the mineralocorticoid receptor

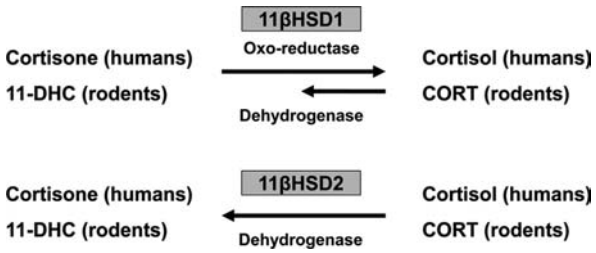

Figure 1. Interconversion of cortisol and cortisone as well as CORT and 11-DHC by 11βHSD type 1 and 2.

from cortisol, which binds with a high affinity. Impaired activity of 11βHSD2 has been shown to result in sodium retention, ultimately leading to hypertension [10]. 11βHSD type 1 (11βHSD1) is a bidirectional enzyme expressed in the liver, bone, and adipose tissue, catalyzing the interconversion of cortisone and 11-DHC to cortisol and CORT, respectively. 11βHSD1 has been identified as a therapeutic target, since it is implicated in various clinical conditions such as obesity, metabolic syndrome, and type 2 diabetes mellitus [11]. The maintenance of normal glucocorticoid levels is therefore critical in maintaining hormonal homeostasis.

In this study we investigated the influence of Rooibos consumption on glucocorticoid plasma levels in human test subjects at risk for CVD and in male Wistar rats. The influence of Rooibos on the inactivation of cortisol was further investigated in Chinese hamster ovary (CHO-K1) cells expressing 11βHSD. The influence of rutin was subsequently investigated at cellular level in the H295R cell model.

2 Materials and methods

2.1 Materials and reagents

Unfermented Rooibos was provided by the South African Rooibos Council (Rooibos LTD-BPK, Clanwilliam, South Africa). Nucleobond® AX plasmid preparation kits were purchased from Machery-Nagel (Duren, Germany). Mirus TransIT®-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Penicillin–streptomycin, fetal calf serum, and trypsin–EDTA were obtained from Gibco BRL (Gaithersburg, MD, USA). Deuterated cortisol (9,11,12,12-D4-cortisol) was purchased from Cambridge isotopes (Andover, MA, USA). Steroids, rutin, forskolin, Dulbecco's modified Eagle's Medium (DMEM), and an MTT assay kit were purchased from Sigma-Aldrich (St. Louis, MA, USA). DMEM/F_{12} and gentamicin were purchased from Invitrogen/Gibco (Grand Island, NY, USA). Cosmic calf serum was supplied by HyClone®, Thermo Scientific (South Logan, UT, USA). A bicinchoninic acid (BCA) protein determination kit was purchased from Pierce (Rockford, IL, USA). The UPLC BEH C18 column was purchased from Waters (Milford, MA, USA). The Kinetex PFP column and SPE columns (Strata-X 33μ Polymetric Reversed Phase, 200 mg/3 mL) were obtained from Phenomenex (Torrance, CA, USA).

All other chemicals were of the highest grade and supplied by trustworthy scientific supply houses.

2.2 Methanolic extractions of unfermented Rooibos

Unfermented Rooibos plant material (30 g) was extracted with chloroform (300 mL) for 8 h using a glass soxhlet extractor fitted with a double wall condenser, followed by a methanol (300 mL) extraction for 8 h. Extracts were protected from light and oxygen at all times to avoid any compositional changes. The methanolic extract was dried on a rotary evaporator and the vacuum released under nitrogen. One gram of Rooibos leaves yielded 0.158 g extract. The dried extract was resuspended in deionized water, centrifuged at 6000 × *g* for 5 min and stored in aliquots at −20°C. Extracts were prepared at a concentration of 20 mg dried extract/mL for in vitro assays and at a concentration of 160 mg dried extract/mL for administration to rats. The extracts were subsequently analyzed using the HPLC-DAD method as previously published by Beelders et al. [12].

2.3 Preparation of human blood samples

Plasma samples were obtained from the study conducted by Marnewick et al. [13]. In this study, the effect of Rooibos was assessed on oxidative stress in 40 adult subjects at risk for CVD. Briefly, the study included 24 females and 16 males between the ages of 30 and 60 years, with at least two or more risk factors for coronary heart disease that included hypercholesterolemia, hypertension, or an increased BMI, while not being medicated. A single group intervention design was followed, consisting of two intervention periods. Subjects first entered a 2-week washout period (control), during which food and beverages with high flavonoid content were eliminated, followed by a 6-week intervention period (Rooibos intervention) in which the same subjects consumed six cups of fermented Rooibos per day (15 g Rooibos leaves/subject). Fasting (10 AM-12 AM) blood samples were collected on completion of each period and centrifuged at 1000 × *g* for 10 min at 4°C. The plasma samples (500 μL) were subsequently diluted with deionized water (1:1), followed by the addition of 15 ng D4-cortisol. Diluted plasma samples, 1 mL, were vortexed with 1 mL ACN and 4 mL ethyl acetate for 10 min. Phases were separated by centrifugation at 500 × *g* for 10 min, the organic phase removed and evaporated under nitrogen. The dried residue was resuspended in 250 μL methanol, followed by the addition of 1 mL deionized water prior to SPE of steroid metabolites. Plasma samples were applied to Strata-X SPE columns, preconditioned with 3 mL methanol and 3 mL deionized water. Columns were washed with 3 mL deionized water and 3 mL 30% methanol. Steroids were eluted with 1 mL methanol and the eluent dried under nitrogen. The dried residue of each sample was resuspended in 150 μL methanol and stored at −20°C until ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis.

2.4 Rat study

This study was cleared the by the University of Stellenbosch Animal Research Ethics Committee (reference nr. 2009B02007) and conformed to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 85–23, revised 1996). Twenty adult male Wistar rats were housed in standard rat cages (5 rats/cage) and fed standard rat chow and tap water ad libitum. Conditions were kept at 21°C with lights set to a 12:12-h reversed light-dark cycle (lights on at 7 PM). Rats were divided into two weight-matched experimental groups ($n = 10$ each) assigned as follows: (i) control group, subjected to two daily placebo treatments (1 mL water/kg body mass) for a period of 10 consecutive days, administered via oral gavage; (2) Rooibos group, maintained as the control group, receiving Rooibos treatments instead of placebo treatments. Methanolic extracts of unfermented Rooibos were prepared as described in Section 2.2. Rats received a dosage equivalent, in terms of soluble solids, to the Rooibos infusion consumed by human subjects (15 g Rooibos leaves/subject daily). A dose translation factor of 6.16 [14] was used to calculate the rat dosage of 0.25 g Rooibos leaves/rat daily. The rats were administered twice the calculated dose due to the Rooibos leaf extract retaining moisture when dried on the rotary evaporator. The rats, with an average mass of 250 g, thus received a total of 0.5 g Rooibos leaves/rat (80 mg Rooibos extract/rat), which was administered twice daily in a dosage of 0.25 g Rooibos leaves/rat (40 mg Rooibos extract/rat) in a volume of 250 μL per gavage. Prior to the intervention protocol, rats were accustomed to the investigator by handling and weighing the animals twice daily (7 AM and 7 PM), 7 times per week for 4 weeks. During the experimental protocol, rats were weighed once daily (7AM), and received two oral treatments daily (7 AM and 7 PM) for 10 consecutive days. On the 11th day of the intervention, rats were sacrificed via decapitation between 8 AM and 11 AM. Trunk blood was collected via a heparinized funnel into lithium heparin tubes and centrifuged at 1000 × *g* for 10 min at 4°C. Plasma samples, 1.5 mL, were subsequently diluted as described in Section 2.3, and following the addition of 15 ng D4-cortisol, the samples were incubated for 1 h at 60°C. Samples were centrifuged at 1000 × *g* for 10 min at 4°C, after which steroids were isolated from the resulting supernatant using SPE as described in Section 2.3.

2.5 Enzyme assays in transiently transfected CHO-K1 cells

CHO-K1 cells were grown at 37°C and 5% CO_2 in DMEM containing 0.9 g/L glucose, 0.12% $NaHCO_3$, 10% fetal bovine serum, and 1% penicillin–streptomycin. Cells were plated into 12 well plates (1 mL/well, 1 × 10^5 cells/mL)

24 h prior to transfection. CHO-K1 cells were transiently cotransfected with a total of 1 μg DNA (0.5 μg human 11βHSD1/pCR3 and 0.5 μg human hexose-6-phosphate dehydrogenase (H6PDH)/pcDNA3.2 or 0.5 μg pCIneo with no DNA insert) using 3 μL Mirus TransIT®LT1 transfection reagent per mL according to the manufacturer's instructions. H6PDH was added to stimulate the oxoreductase activity of 11βHSD1. CHO-K1 cells were also cotransfected with 0.5 μg human 11βHSD2/pCR3 plasmid DNA and 0.5 μg pCIneo, using the same protocol. Cells were incubated for 72 h after which cortisol (1 μM) and cortisone (1 μM) were added to the medium. 11βHSD1 and 11βHSD2 activities were assayed in the absence and presence of Rooibos by the addition of 50 μL unfermented Rooibos extract (final concentration, 1 mg dried extract/mL). After 24 h, the medium (500 μL) was removed and steroids extracted from the media using a 10:1 volume of dichloromethane to culture medium. The mixture was vortexed for 2 min, centrifuged at 500 × *g* for 5 min. The dichloromethane phase was dried under nitrogen, resuspended in 150 μL methanol and stored at −20°C until UPLC-MS/MS analysis. After each assay the cells were washed with PBS (10 mM, pH 7.4), collected in the same buffer and sonicated prior to protein determination by the Pierce BCA® method.

2.6 Steroid metabolism in H295R cells

H295R cells were grown to confluency at 37°C and 5% CO_2 in growth medium (DMEM/F_{12}, supplemented with L-glutamine, 15 mM HEPES, pyridoxine, 1.125 g $NaHCO_3$/L, 1% penicillin streptomycin, 0.01% gentamicin, and 10% cosmic calf serum). Steroid metabolism was assayed as follows: Cells were plated into 12 well plates (1 mL/well, 4×10^5 cells/mL) and incubated for 48 h. The medium was subsequently replaced with experimental medium (growth medium containing 0.1% cosmic calf serum) and cells were incubated for 12 h after which the appropriate treatments were added in experimental medium. Steroid metabolism was assayed in the presence of Rooibos, by the addition of 50 μL extract per well (final concentration, 1 mg dried extract/mL) and in the presence of rutin, added to a final concentration of 10 μM, under basal and forskolin (10 μM) stimulated conditions. After 48 h, the medium (500 μL) was removed and 15 ng D4-cortisol was added as an internal standard. Steroids were extracted and the protein content determined as described in Section 2.5. After each assay, the cells were washed with, and collected in PBS (10 mM, pH 7.4), and sonicated prior to protein determination by the Pierce BCA® method.

2.7 UPLC-MS/MS separation and quantification of steroid metabolites

Steroid metabolites from human plasma, and conversion assays in CHO-K1 and H295R cells, were separated by UPLC (ACQUITY UPLC, Waters, Milford, MA, USA) using a Phenomenex UPLC Kinetex PFP (2.1 mm × 100 mm, 2.6 μm) column as previously described [8]. Steroid metabolites from rat plasma were separated by UPLC (ACQUITY UPLC) using a Waters UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm) column as previously described by Storbeck et al. [15]. A Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was used for quantitative mass spectrometric detection. All steroids were analyzed in multiple reaction monitoring mode using an electrospray in the positive ionization mode (ESI+). The following settings were used: capillary voltage of 2.8 kV, cone voltage 15–50 V, collision energy 3–32 eV, source temperature 100°C, desolvation temperature 500°C, desolvation gas 1000 L/h and cone gas 50 L/h. Calibration curves were constructed using weighted ($1/x2$) linear least squares regression. Data were collected with the MassLynx 4.1 software program.

2.8 Cell viability

Confluent CHO-K1 cells were plated out in a 96 well plate (100 μL, 1×10^5 cells/mL) and incubated with Rooibos extract (1 mg/mL) for 24 h. Confluent H295R cells were plated out in a 96 well plate (100 μL, 4×10^5 cells/mL) and incubated with Rooibos extract (1 mg/mL), rutin (10 μM), and forskolin (10 μM) for 48 h. Cell viability was subsequently assayed using an MTT toxicology assay kit according to the manufacturer's instructions. A color-standardized control (media containing 1 mg/mL Rooibos extract) was included to compensate for the possible interference of the Rooibos color with the assay. The following absorption values were obtained: cells exposed to media only (ΔA, 0.335), cells exposed to media containing 1 mg/mL Rooibos extract (ΔA, 0.334) and cells exposed to media containing 10 μM rutin (ΔA, 0.305). Since Rooibos and rutin did not have a significant influence on cell viability, the effects of these compounds on steroid production in vitro were assayed at abovementioned concentrations.

2.9 Statistical analysis

Data from in vivo studies are summarized as boxplots to illustrate and compare the distribution of the steroid hormone levels for rats, stratified by group (control or +Rooibos) and in humans, stratified by treatment (control or +Rooibos) within gender. Boxplots show the minimum, maximum (whiskers), first and third quartiles (bottom and top of box) and median (line inside box) of each group. If there were outliers, they are depicted as dots and the whiskers extended to the next ordered value. Some distributions were skewed, illustrated by the outliers, and therefore those outcomes (testosterone, CORT:testosterone, cortisol:cortisone, cortisol:testosterone and cortisol:DHEA-S) were log-transformed to approximate normality, prior to analysis, for validity. The association of age with basic characteristics, after adjusting for gender, were also tested. General linear models were used to compare variables

Table 1. Major phenolic compounds (μg) present in the methanolic extract of unfermented Rooibos plant material and in the extract administered to rats and cells

Flavonoid compounds	Rooibos[a)]	Rats[b)]	Cells[c)]
Aspalathin	23 560.0 ± 1035.0	11 930.0 ± 523.8	149.1 ± 6.5
Nothofagin	4915.0 ± 408.7	2488.0 ± 206.9	31.1 ± 2.6
Rutin	621.9 ± 28.1	314.9 ± 14.3	3.9 ± 0.2
Quercetin-3-*O*-robinobioside	1138.0 ± 53.6	576.1 ± 27.1	7.2 ± 0.3
Orientin	2356.0 ± 61.2	1193.0 ± 30.9	14.9 ± 0.4
Isoorientin	3153.0 ± 17.7	1597.0 ± 8.9	20.0 ± 0.1
Vitexin	435.7 ± 27.6	220.6 ± 14.0	2.8 ± 0.2
Isovitexin	477.7 ± 25.7	242.1 ± 13.2	3.0 ± 0.2

Values are expressed as the mean ± SEM.
a) Flavonoids extracted from 1 g unfermented Rooibos leaves (methanolic extraction).
b) Flavonoids present in Rooibos extract administered per rat per day.
c) Flavonoids present in Rooibos extract administered to CHO-K1 cells (24 h) and H295R cells (48 h) per mL media.

between groups of rats as well as general characteristics of the human group between genders. Since the same human subjects were tested for both the control and Rooibos intervention period, the correlation between these pairs of measurements were taken into account by including random effects for individuals in mixed-effects linear models. We tested for gender differences in the effect of Rooibos (statistical interaction between gender and Roooibos group) and where there was no interaction, we tested for difference between Rooibos groups, adjusted for gender. All *p*-values, effect sizes, and confidence intervals reported are from these models. In vivo data were analyzed in base R and R package nlme, freely available from www.r-project.org. The data from CHO-K1 cells were analyzed with an unpaired *t*-test, while the H295R data were analyzed with a one-way ANOVA, followed by a Newman–Keuls multiple comparison test using GraphPad Prism (version 5) (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Analysis of methanolic extracts of unfermented Rooibos

The methanolic extract of unfermented Rooibos used in the rat study and in our in vitro experiments (H295R and CHO-K1 cells) was analyzed for polyphenols using HPLC-DAD as previously published [12]. Quantification of the major phenolic compounds (Table 1) showed that, per gram unfermented Rooibos leaves, the dihydrochalcones were the most abundant flavonoids present in the extract, with aspalathin being 4.8-fold higher than nothofagin. Within the group of flavones, isoorientin, and orientin levels were considerably higher (6-fold) than those of vitexin and isovitexin. Analyses of the flavonols showed that the rutin isomer, quercetin-3-*O*-robinobiocide was present at levels 1.8-fold higher than that of rutin. The rats, with an average mass of 250 g, each received a total of 18.56 mg of the major phenolic compounds daily, while the final concentration of these compounds ranged from 6.4 to 330 μM in the media to which the CHO-K1 and H295R cells were exposed.

In order to ascertain the major phenolic compounds present in fermented Rooibos, these compounds were subsequently also quantified in a methanolic extract of fermented Rooibos as well as aqueous extracts of fermented and unfermented Rooibos (Supporting Information Table 1). The analyses show that the greatest difference between the aqueous extract of fermented Rooibos and the methanol extract of unfermented Rooibos lies in aspalathin and nothofagin levels being higher, 45.7-fold and 38.9-fold, respectively, in unfermented Rooibos. The flavones, orientin, isoorientin, vitexin, and isovitexin were ± 2.0-fold higher in unfermented Rooibos, while the flavonols were also detected at higher levels, rutin (5.5-fold), and quercetin-3-*O*-robinobiocide (1.6-fold).

3.2 Analysis of plasma steroid levels in human subjects following Rooibos consumption

Human plasma was analyzed for the levels of cortisol, cortisone, dehydroepiandrosterone-sulfate (DHEA-S) and testosterone. The effect of Rooibos on cortisol (Fig. 2A), testosterone (Fig. 2C), and DHEA-S (Fig. 2D) levels in both male and female subjects did not reach statistical significance. Rooibos consumption did, however, significantly increase the levels of cortisone in males by 9.1 nmol/L (95% CI: 1.3–17.0 nmol/L; $p = 0.0465$) but not in females (Fig. 2B). The cortisol:cortisone ratio, however, was significantly lower in both male and female subjects following Rooibos consumption, resulting in an estimated reduction of 6.7% (95% CI: 0-12.9%; $p = 0.0486$) after adjusting for gender (Fig. 3A). No significant effects were detected on the cortisol:testosterone (Fig. 3B) or cortisol:DHEA-S (Fig. 3C) ratios in male or female subjects.

3.3 Analysis of plasma steroid levels in male Wistar rats following Rooibos consumption

Rat plasma was analyzed for the levels of CORT, DOC, 11-DHC, and testosterone. A significant reduction of

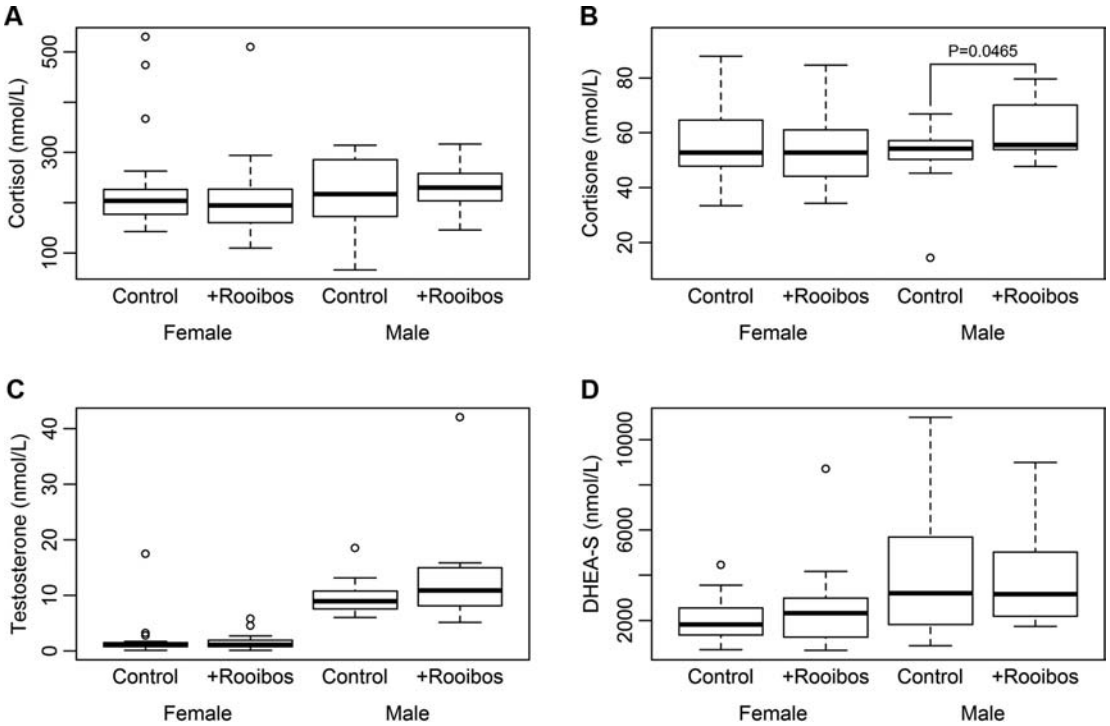

Figure 2. Boxplots of human plasma (A) cortisol, (B) cortisone, (C) testosterone, and (D) DHEA-S levels in male and female subjects in the absence (control) and presence of Rooibos (+Rooibos). The estimated effect of Rooibos on cortisone was an increase of 9.1 nmol/L (95% CI: 1.3–17.0 nmol/L) in male subjects only. No other significant effects were detected.

131.6 nmol/L was observed in CORT plasma levels of rats receiving Rooibos treatments (95% CI: 16.3–246.8 nmol/L; $p = 0.0275$) compared to the control group (Fig. 4A). In addition, plasma DOC levels were 1.75 nmol/L lower in rats consuming Rooibos (95% CI: 0.19–3.32 nmol/L; $p = 0.0298$; Fig. 4B). The influence of Rooibos on the levels of 11-DHC (Fig. 4C) and testosterone (Fig. 4D) did not reach statistical significance. Although Rooibos only decreased the CORT:11-DHC ratio to a level approaching significance ($p = 0.0624$; Fig. 5A), it caused a 57% reduction in the CORT:testosterone ratio (95% CI: 32–72%; $p = 0.0009$; Fig. 5B).

3.4 Cortisol and cortisone conversion assays by 11βHSD in transiently transfected CHO-K1 cells

The influence of Rooibos (1 mg extract/mL) on the interconversion of cortisol and cortisone by 11βHSD1 as well as on the conversion of cortisol to cortisone by 11βHSD2 was assayed in CHO-K1 cells. 11βHSD1 was assayed with and without H6PDH, the cofactor regenerating system that stimulates the oxoreductase activity of 11βHSD1. After a 24 h incubation period with cortisone (reductase/forward reaction) in cells co-expressing 11βHSD1 and H6PDH, the addition of Rooibos resulted in a significant reduction (68%, $p < 0.01$) in the cortisol:cortisone ratio (Fig. 6A). After the same incubation period with cortisone in cells expressing 11βHSD1 alone, the addition of Rooibos also resulted in a significant decrease in the cortisol:cortisone ratio (56%, $p < 0.01$) even though the ratio of cortisol:cortisone in the absence of Rooibos was lower than that in cells co-expressing 11βHSD1 and H6PDH. The increased cortisol:cortisone ratios in the presence of H6PDH is due to stimulated oxoreductase activity, resulting in increased levels of cortisol. The same experiment was carried out using cortisol as substrate to assay the dehydrogenase/reverse reaction of 11βHSD1. After 24 h, Rooibos significantly reduced the cortisol:cortisone ratio in cells co-expressing 11βHSD1 and H6PDH (39%, $p < 0.01$) as well as in cells expressing 11βHSD1 only (33%, $p < 0.01$; Fig. 6B). No effect was observed on the enzymatic activity of 11βHSD2 when the cortisol:cortisone ratio was determined in the presence of Rooibos (Fig. 6C).

3.5 Analysis of steroid metabolites in adrenal H295R cells

The effect of rutin (10 μM) on steroid metabolism in H295R cells was investigated (Table 2) and compared with the effect previously observed in the presence of Rooibos (1 mg/mL extract) under basal and forskolin-stimulated (10 μM) conditions [8]. While Rooibos significantly reduced the levels of deoxycortisol ($P < 0.01$), DHEA-S ($p < 0.01$), A4 ($p < 0.01$), 11βOHA4 ($p < 0.01$) and testosterone ($p < 0.001$) under basal conditions, the effect of rutin was negligible. Upon forskolin stimulation, however, Rooibos significantly decreased the

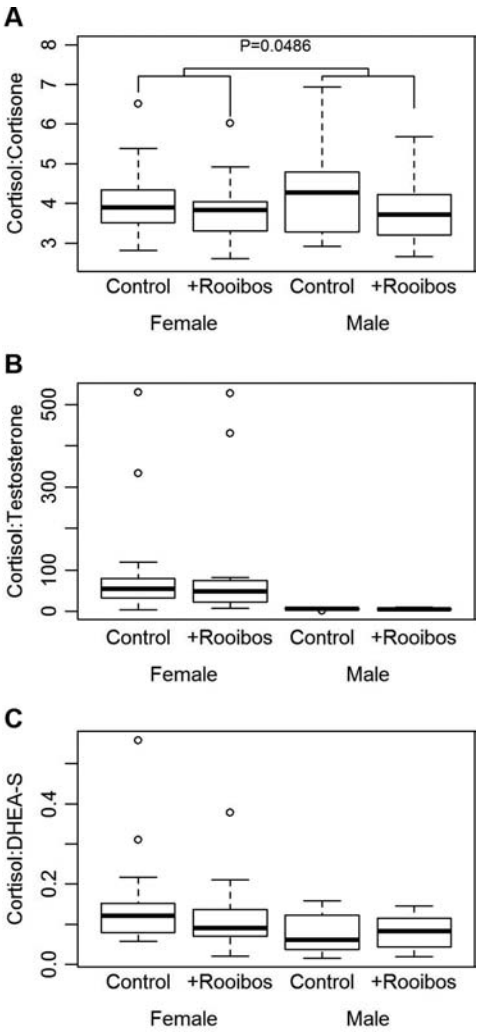

Figure 3. Boxplots of human plasma (A) cortisol:cortisone, (B) cortisol:testosterone and (C) cortisol:DHEA-S ratios in male and female subjects in the absence (control) and presence of Rooibos (+Rooibos). The estimated effect of Rooibos on the cortisol:cortisone ratio was a decrease of 6.7% (95% CI: 0–12.9%) in both genders. No other significant effects were detected.

levels of DOC ($p < 0.01$), deoxycortisol ($p < 0.001$), CORT ($p < 0.001$), cortisol ($p < 0.001$) and cortisone ($p < 0.01$), while rutin significantly reduced the levels of deoxycortisol ($p < 0.01$), CORT ($p < 0.05$) and cortisol ($p < 0.001$). Rooibos and rutin both reduced the levels of A4 ($p < 0.001$), 11βOHA4 ($p < 0.001$) and testosterone ($p < 0.001$) under stimulated conditions, however, the levels of DHEA-S was reduced in the presence of rutin only ($p < 0.001$).

4 Discussion

Fermented Rooibos, which exhibits significantly lower antioxidant activity and contains lower levels of aspalathin and nothofagin than unfermented Rooibos [6], was consumed for 6 weeks during the human study, after which we analyzed plasma steroid levels. Unfermented Rooibos was used in the rat study and in vitro experiments and analyses of the major polyphenols in our methanolic extract (Table 1) detected significantly higher levels of aspalathin, nothofagin and rutin, while the other flavonoids analyzed were ± 2-fold higher than the compounds in the aqueous extract of fermented Rooibos (Supporting Information Table 1). It should be noted that the aqueous extract was prepared with a different batch of fermented Rooibos as that which was consumed in the human study. While it can be assumed that aspalathin and nothofagin levels would differ most significantly to that of unfermented Rooibos, there may also be variation in fermented Rooibos samples due to factors such as the product originating from different plant material, the fermentation and drying process, as well as natural variation [12]. Interestingly, when comparing the aqueous extract and the methanolic extract of unfermented Rooibos, the flavone extraction was higher in the organic extract. Although the aglycones were not analyzed, these hydrophobic compounds may also be present in the methanolic extract, however, it is also possible that they were removed in the chloroform fraction during the extraction process. Comparisons of fermented and unfermented Rooibos showed that aspalathin and nothofagin levels are significantly reduced in fermented Rooibos, as has also been reported by other groups [6, 12].

We first analyzed the effect of Rooibos consumption in vivo—on circulating glucocorticoid levels, cortisol and its inactive keto-metabolite, cortisone, in the human study. Our analyses showed that cortisone levels increased significantly ($p = 0.0465$) in male subjects following Rooibos consumption (Fig. 2B), while the effect on cortisol levels in both male and female participants was negligible (Fig. 2A). Since in vivo animal studies have fewer limitations and are conducted under more controlled and standardized conditions than is possible for human studies, the influence of Rooibos on adrenal steroidogenesis was further investigated in male Wistar rats, minimizing the risk of effects being masked by confounding factors. Due to the lack of adrenal cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) expression, CORT is the principal plasma glucocorticoid in rats [9] and the measurement thereof is generally used an indicator of stress. Plasma levels of CORT ($p = 0.0275$; Fig. 4A) and its precursor metabolite, DOC ($p = 0.0298$; Fig. 4B), were reduced significantly in rats receiving Rooibos, compared to the control group. The effect of Rooibos on the inactive metabolite of CORT, 11-DHC (Fig. 4C), however, did not reach statistical significance. The reduction observed in CORT and DOC levels indicates that Rooibos reduced glucocorticoid production in rats, likely due to inhibition of CYP11B1, and possibly upstream steroidogenic enzymes such as 3βHSD and cytochrome P450 21-hydroxylase (CYP21A2). We have previously shown that Rooibos extracts inhibit CYP17A1 and CYP21A2 [8], while a study by Ohno et al. [7], showed that 3βHSD and CYP21A2 were more sensitive to an array of flavonoid compounds when compared to other adrenal steroidogenic enzymes.

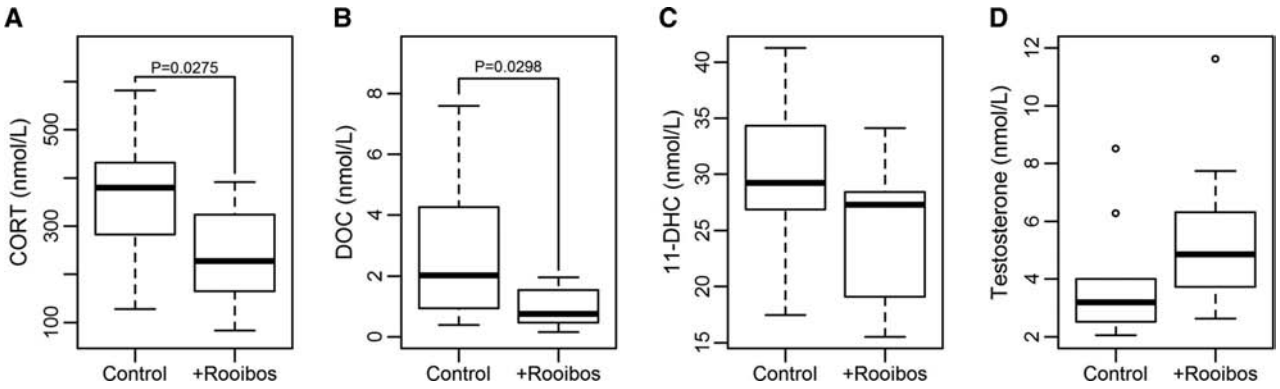

Figure 4. Boxplots of plasma (A) CORT, (B) DOC, (C) 11-DHC and (D) testosterone levels in male Wistar rats in the absence (control) and presence of Rooibos (+Rooibos). The estimated effect of Rooibos on CORT was a decrease of 131.6 nmol/L (95% CI: 16.3–246.8 nmol/L) and an estimated decrease of 1.75 nmol/L (95% CI: 0.19–3.32 nmol/L) was observed on the levels of DOC. No other significant effects were detected.

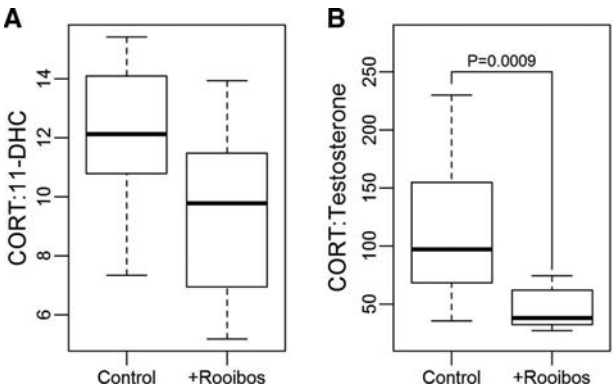

Figure 5. Boxplots of plasma (A) CORT:11-DHC and (B) CORT:testosterone ratios in male Wistar rats in the absence (control) and presence of Rooibos (+Rooibos). The estimated effect of Rooibos on the CORT:testosterone ratio was a decrease of 57% (95% CI: 32–72%). No other significant effects were detected.

We subsequently analyzed the effect of Rooibos on steroid metabolite ratios, since ratios rather than absolute concentrations alone are generally used to better characterize clinical conditions linked to stress related diseases. Cortisol:cortisone (humans) and CORT:11-DHC (rats) ratios in circulating blood reflect the interconversion of active glucocorticoids and their inactive keto-metabolites by 11βHSD1 and 11βHSD2. In the human study, a significant reduction in the cortisol:cortisone ratio of both male and female subjects ($p = 0.0486$) was detected following Rooibos consumption (Fig. 3A), suggesting that Rooibos favors the inactivation of cortisol. It is possible that the reduced cortisol:cortisone ratio could be attributed to altered 11βHSD activity, since in vivo cortisol levels remained within the normal range. Significantly decreased cortisol levels may lead to an increase in the activity of the HPA axis and undesired stimulation of the adrenal by ACTH to produce cortisol [16]. In rats, the CORT:11-DHC ratio decreased to a level approaching significance ($p = 0.0624$) following Rooibos consumption (Fig. 5A). Although Rooibos significantly reduced rat CORT levels, the effect on 11-DHC was negligible, possibly contributing to lowering the effect observed on the CORT:11-DHC ratio.

The reduction observed in the cortisol:cortisone ratio in human subjects following Rooibos consumption prompted us to examine the effect of Rooibos on glucocorticoid inactivation by the two 11βHSD isozymes at cellular level. Co-transfection of 11βHSD1 and H6PDH in CHO-K1 cells simulates in vivo conditions in which the oxoreductase activity of 11βHSD1 predominates, while virtually eliminating the dehydrogenase activity [17]. High ratios of NADPH:NADP, required for the oxoreductase activity of 11βHSD1, are maintained by H6PDH, which converts glucose-6-phosphate to 6-phospho-gluconolactone, regenerating NADPH from NADP [18]. Enzyme activity was assayed in the absence and presence of Rooibos extract (1 mg/mL) using either 1 μM cortisone or 1 μM cortisol as substrate. The addition of H6PDH resulted in a 1.7-fold increase in the oxoreductase activity, eliminating the dehydrogenase activity completely, with no cortisone being detected after 24 h (results not shown), which is in agreement with data obtained by Bujalska et al. [17]. In the present study, Rooibos significantly reduced the cortisol:cortisone ratio in cells expressing 11βHSD1, both in the absence and presence of H6PDH, suggesting that Rooibos modulates 11βHSD1 and does not interfere with H6PDH and the cofactor regenerating system. From the data it is clear that Rooibos reduces the cortisol:cortisone ratio when either cortisone (Fig. 6A) or cortisol (Fig. 6B) conversion is assayed in cells expressing 11βHSD1, in the absence and presence of H6PDH. 11βHSD2 activity was, however, unaffected by Rooibos with no effect on the cortisol:cortisone ratio being detected after 24 h (Fig. 6C). It is widely reported that 11βHSD1 plays a pivotal role in the regulation of glucocorticoid action, with dysregulation and subsequent long-term glucocorticoid excess being strongly associated with numerous clinical conditions linked to the metabolic syndrome [19]. Selective 11βHSD1 inhibitors tested in rodents have been

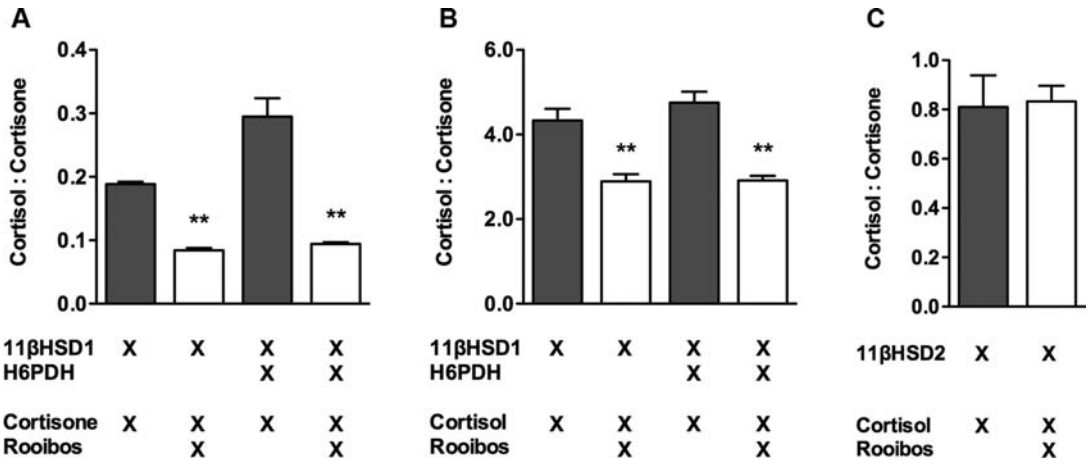

Figure 6. Cortisol:cortisone ratios after 24 h in CHO-K1 cells transiently transfected with 11βHSD1 and 11βHSD2. 11βHSD1 expression, with and without H6PDH, was assayed with (A) 1 μM cortisone as substrate and (B) 1 μM cortisol as substrate, while 11βHSD2 expression was assayed with (C) 1 μM cortisol as substrate, in the absence and presence of Rooibos extract (1 mg/mL). The data was analyzed with an unpaired *t*-test and results are expressed as the mean ± SEM (**$p < 0.01$). The data is representative of two independent experiments, performed in triplicate.

shown to increase insulin sensitivity and decrease endogenous glucose production, together with a reduction in body mass and circulating lipid levels [18]. It is therefore evident that 11βHSD1 inhibitors may play a key role in the treatment of metabolic syndrome and type 2 diabetes.

Since flavonoids have been shown to modulate the activity of P450 enzymes and affect steroid hormone biosynthesis [7, 20], we compared the effect of rutin, 10 μM, on steroid metabolism in H295R cells with the effect previously observed in the presence of 1 mg/mL Rooibos extract, which contained rutin at 3.9 μg/mL (6.4 μM), and quercetin-3-*O*-robinobiocide at 7.2 μg/mL (11.8 μM) (Table 1). Rutin and its isomer are reported to form part of the major phenolic compounds present in Rooibos, however, there is marked variation between Rooibos production batches [21, 22], with our analyses also showing rutin to be present in higher levels in unfermented than in fermented aqueous extracts of Rooibos (Supporting Information Table 1). Analysis of the Rooibos extract added to the cells, showed that the cells were exposed to ± 0.5 mM flavonoids. These flavonoids however, represent only a fraction of the total phenolic compounds present in Rooibos.

The H295R cell line is an excellent model system for studying the effect of nutraceuticals, dietary supplements and food additives on steroid hormone biosynthesis, since these cells express all the steroidogenic enzymes and are capable of producing the mineralocorticoids, glucocorticoids, and adrenal androgens. These cells can also be manipulated by the addition of forskolin, a diterpene that mimics the effects of ACTH via the activation of adenyl cyclase (cAMP) pathways in adrenal cells, thus imitating a stress response [23]. Analyses of the total steroid output showed that the influence of rutin was not as pronounced as that of Rooibos under both basal and stimulated conditions. Rutin did not influence basal steroid production significantly, however, upon forskolin stimulation, rutin significantly reduced the total steroid output from 11.1 to 4.9 μM ($p < 0.01$). While neither rutin nor Rooibos inhibited basal CORT or cortisol production significantly, upon stimulation, rutin reduced the levels of CORT ($p < 0.05$) and cortisol ($p < 0.001$), however, markedly less than the inhibitory effect observed in the presence of Rooibos (Table 2). Rutin also significantly reduced the levels of deoxycortisol ($p < 0.01$) under stimulated conditions, however not to the same extent as Rooibos ($p < 0.001$). Regarding the inactive glucocorticoid metabolites, cortisone and 11-DHC, the effect of both Rooibos and rutin was negligible under basal conditions, however, nonsignificant decreases in 11-DHC and cortisone production were observed upon forskolin stimulation. However, the contribution of the adrenal toward circulating levels of inactive glucocorticoid metabolites is negligible, since the expression of the 11βHSD isoforms is low in the adrenal [24]. From these results it is clear that rutin inhibits glucocorticoid production, as well as the respective precursor metabolites under stimulated conditions. Interestingly, while rutin decreased cortisol levels significantly in the presence of forskolin, the effect of Rooibos on cortisol levels was more prominent, reducing cortisol levels to the levels detected under basal conditions.

We previously showed that Rooibos not only reduced the flux through the glucocorticoid pathway, but also through the androgen pathway in forskolin stimulated H295R cells, prompting us to analyze the plasma levels of testosterone and DHEA-S in our in vivo investigations. In addition, we also reported that Rooibos inhibited CYP17A1, expressed in COS-1 cells, possibly accounting for the decreased androgen levels observed in H295R cells [8]. It must be noted that, while the testes is the major site of testosterone production in males, the adrenal also produces low levels of testosterone in both males

Table 2. Steroid metabolism in H295R cells under basal and forskolin (10 μM) stimulated conditions in the presence of Rooibos (1 mg/mL) and rutin (10 μM) after 48 h

Steroids	Basal[a)] Steroid levels (nM)	+Rooibos[a)] Steroid levels (nM)	+Rutin Steroid levels (nM)
DOC	107.4 ± 13.0	54.0 ± 8.0	125.7 ± 7.0
Deoxycortisol	1741.0 ± 234.1	506.4 ± 23.0**	1998.0 ± 128.9
CORT	241.1 ± 31.6	144.9 ± 13.5	280.2 ± 34.6
Cortisol	670.2 ± 39.3	381.7 ± 63.7	550.3 ± 28.1
11-DHC	9.9 ± 0.3	9.0 ± 0.9	11.8 ± 1.4
Cortisone	6.6 ± 2.2	4.9 ± 0.3	6.0 ± 0.3
DHEA-S	3.5 ± 0.3	9.1 ± 1.0**	5.5 ± 1.1
A4	806.5 ± 115.6	307.4 ± 27.5**	890.9 ± 74.4
11βOHA4	90.1 ± 11.1	162.7 ± 10.6**	83.0 ± 10.7
Testosterone	39.22 ± 3.60	4.79 ± 0.70***	44.70 ± 3.49
Steroids	**Forskolin[a)] Steroid levels (nM)**	**Forskolin + Rooibos[a)] Steroid levels (nM)**	**Forskolin + Rutin Steroid levels (nM)**
DOC	343.2 ± 61.1	104.0 ± 11.0**	510.1 ± 85.8
Deoxycortisol	2757.0 ± 393.1	539.8 ± 26.8***	1409.0 ± 156.0**
CORT	2062.0 ± 170.5	394.9 ± 27.2***	1267.0 ± 88.8*
Cortisol	3793.0 ± 285.1	772.1 ± 40.5***	1246.0 ± 32.3***
11-DHC	11.2 ± 1.4	8.4 ± 1.4	7.1 ± 1.8
Cortisone	11.6 ± 2.7	2.3 ± 0.4**	5.7 ± 2.7
DHEA-S	5.2 ± 0.5	3.8 ± 0.5	1.2 ± 0.6***
A4	1402.0 ± 180.0	259.3 ± 22.6***	321.2 ± 34.6***
11βOHA4	388.3 ± 16.3	167.0 ± 5.7***	57.9 ± 7.0***
Testosterone	50.5 ± 6.1	2.4 ± 0.5***	8.9 ± 1.0***

Steroids are expressed as absolute values (nmol/L). Data was analyzed by a one-way ANOVA, followed by a Dunnett's multiple comparison test and results are expressed as the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The data is representative of two independent experiments, performed in triplicate.
a) Data regarding the influence of Rooibos on basal and forskolin stimulation in H295R cells was reproduced and modified from Schloms et al. 2012, with permission from Elsevier.

and females due to the expression of 17β-hydroxysteroid dehydrogenase type 3 and 5 [24]. In the human study, Rooibos consumption did not affect male or female testosterone (Fig. 2C) and DHEA-S (Fig. 2D) levels significantly. Since rat adrenals do not produce DHEA-S [9], we only analyzed testosterone levels, which showed that the effect of Rooibos consumption on these levels did not reach statistical significance (Fig. 4D). In a study by Rosmund et al. [25], it was shown that altered cortisol:testosterone ratios are prevalent in clinical conditions such as type 2 diabetes, hypertension, and CVD. In addition, Smith et al. [26] showed that elevated circulating cortisol:testosterone ratios are associated with an increased risk of ischemic heart disease, mediated through insulin resistance. The authors therefore hypothesized that agents capable of reducing the cortisol:testosterone ratio may improve insulin resistance and lower the risk of coronary heart disease. In the human study, Rooibos consumption had no significant effect on the cortisol:testosterone ratio in male or female subjects (Fig. 3B), however, in the rat study, Rooibos consumption significantly reduced the CORT:testosterone ratio by 57% ($p = 0.0009$; Fig. 5B). We also studied the effect of Rooibos on the cortisol:DHEA-S ratio in the human study, since it was recently shown that this ratio is positively associated with the metabolic syndrome [27]. Analyses of human plasma showed that the effect of Rooibos on the cortisol:DHEA-S ratio did not reach statistical significance, even though the subjects were at risk for CVD, with negligible differences being observed after the intervention period (Fig. 3C). The lack of statistical power to detect significance in steroid levels in human plasma could be attributed to the small number of participants in the study. It is also possible that the interindividual variation among the participants regarding steroid levels and age contributed toward lowering the statistical power. Interestingly, under stimulated conditions in H295R cells, both Rooibos and rutin decreased the levels of the adrenal androgens, androstenedione (A4), 11β-hydroxyandrostenedione (11βOHA4), and testosterone significantly ($p < 0.001$), and, in contrast to Rooibos, rutin also significantly decreased DHEA-S levels ($p < 0.001$; Table 2). DHEA-S, which is synthesized primarily in the adrenal, is the major source of testosterone in women, while in men, about half of the total androgen pool is synthesized from these steroids [28]. Under basal conditions in H295R cells, Rooibos also reduced the levels of the androgens, A4 ($p < 0.01$), 11βOHA4 ($p < 0.01$), and testosterone ($p < 0.001$), however, the levels of DHEA-S increased significantly

($p < 0.01$). Rutin had no influence on basal androgen production in H295R cells.

From our data it is clear that rutin did not in all cases result in the same inhibitory effects brought about by Rooibos in the adrenal cell model, indicating that the flavonoid composition of Rooibos results in markedly different inhibitory effects on adrenal steroidogenic enzymes. The H295R cells were, however, exposed to higher levels of flavonoid compounds (Table 1), with aspalathin being present at 330 μM and nothofagin at 71 μM. We previously assayed both compounds, and at 10 μM, both aspalathin and nothofagin decreased 17OHPROG and 11βOHA4 levels, while Rooibos increased basal production of these steroids. We have also determined that aspalathin, nothofagin, rutin, and vitexin, at 10 μM, inhibit CYP17A1 and 3βHSD significantly. Interestingly, the activity of CYP11B1 was inhibited significantly by rutin when deoxycortisol was used as substrate, while vitexin had no influence on the conversion. In contrast, when DOC was added as substrate, vitexin's inhibition was significant while rutin did not affect the reaction (unpublished data). It is evident that the phenolic compounds in Rooibos interact differently with the enzymes catalyzing steroid hormone biosynthesis, and while some may inhibit specific enzymes, others may have no influence, with the effects observed also being substrate dependent. In a recent study by Hasegawa et al. [29], a range of flavone, flavanone, and isoflavone compounds were assayed in H295R cells. Catechin, a monomeric flavanol also present in Rooibos, had no effect on any of the steroid levels assayed, while apigenin increased CYP17A1 products, PREG, DHEA, and 17OHPROG, and inhibited DOC and A4 production. In addition, the compound also decreased CYP17A1, CYP21A2 and 3βHSD mRNA expression in forskolin stimulated H295R cells—effects that were not observed in unstimulated cells [29]. Although this aglycone has not been identified in Rooibos, its glycosylated derivatives, apigenin-6-C-glucoside (isovitexin), and apigenin-8-C-glucoside (vitexin) have been identified [12]. Both vitexin and isovitexin may be converted to apigenin by intestinal microbacteria, with vitexin having been identified in human plasma [22]. It should be noted that, in our assays in H295R cells, in which the cells were exposed to extracts or compounds, the bioavailability and metabolism of the flavonoid compounds were not taken into account, and data obtained as such, may not reflect the effect on the system in vivo. Although the influence of Rooibos on steroidogenesis cannot be attributed to a single flavonoid compound, we showed that aspalathin, nothofagin [8] and rutin are able to influence steroid production in H295R cells. Both rutin and aspalathin have been shown to be present in human plasma in their unchanged forms following Rooibos consumption. However, recovery rates were low and marked interindividual variation in absorption patterns of the human subjects in the study was evident [22]. Rutin has been shown to be metabolized through the action of intestinal bacterial enzymes to quercetin, which may be further metabolized to yield phenolic acids [30] as well as methylated and glucuronidated metabolites [31]. Quercetin may contribute toward the effect of Rooibos on steroid plasma levels, as it has been shown to inhibit 3βHSD activity significantly at 10 μM [7]. While it was subsequently shown to have no significant influence on cortisol production in H295R cells [32], it was shown to increase intracellular cAMP concentrations and to induce aromatase activity [33]. While nothofagin was not detected in plasma samples, it was detected in urine samples, together with aspalathin, as conjugated and as unchanged metabolites [21]. Whether these phenolic compounds reach target tissues in their intact form, at physiologically relevant concentrations, and whether effects are due to their native structure, is perhaps questionable. However, more than 50 phenolic compounds have, to date, been identified in Rooibos [6, 12]. These dietary polyphenols, and their conjugated pool of flavonoids produced by intestinal and colonic microbiota, which may contribute to their bioavailability, as well as postabsorption metabolism by hepatic enzymes, suggest a key role for Rooibos flavonoids in the observed effects on steroidogenesis.

Although the data obtained in this study does place Rooibos in a favorable position in terms of health benefits, not all the polyphenol compounds present in Rooibos are necessarily beneficial. Plant polyphenols are ingested daily not only as part of a normal diet, but also, in many instances, as dietary supplements. Tea flavonoids have gained popularity as added value products in the food supplement and health industries and, being regarded as nutraceuticals, are used to promote polyphenol-rich products. However, increasing the dosage does not necessarily mean that the increase may be beneficial or that more compounds, if any, will reach specific target tissues. Health benefits regarding the ingestion of concentrated polyphenols are being questioned, as limited in vivo data is available on the metabolism and absorption thereof. Papers reporting the beneficial effects of polyphenols, however, far outweigh those reporting on the antinutritional and toxicity aspects of polyphenols. Polyphenols taken in high dosages have been linked to hepatotoxic effects, toxicity, drug interaction as well as estrogenic effects [34], however, these effects have only been reported when high dosages of concentrated dietary supplements or purified tea preparations were taken, which is not the case for the consumption of daily teas or herbal infusions.

Taken together, our data shows that although the subjects in the human study received a lower dose of flavonoid compounds (per kg body mass) due to the consumption of fermented tea, a significant effect on cortisol:cortisone ratios was nevertheless achieved, indicating that the Rooibos consumed resulted in changes in these steroid levels. This effect was also observed in CHO-K1 cells when cortisol:cortisone ratios were analyzed by assaying 11βHSD activity in the presence of unfermented Rooibos. Interestingly, the significant decrease in cortisol levels obtained with unfermented Rooibos in the adrenal cell model was not observed in humans, possibly due to the lower flavonoid intake. In the rats, however, there was a reduction in plasma glucocorticoids, with

CORT and DOC levels being reduced significantly, which was also reflected in the data obtained in stimulated H295R cells in the presence of unfermented Rooibos. While testosterone levels were decreased significantly in H295R cells, these levels remained unchanged in rats, however, a significant decrease was detected in the CORT:testosterone ratio.

From our data it would appear that Rooibos affects glucocorticoid biosynthesis by reducing glucocorticoid production in the adrenal and/or by favoring the inactivation of glucocorticoids to their inactive keto-metabolites by 11βHSD. Although a degree of uncertainty exists with regards to the absorption and bioavailability of the compounds present in Rooibos, the daily consumption of Rooibos is not associated with the ingestion of concentrated doses of polyphenols, and has not been linked to toxic effects. Our in vivo and in vitro studies suggest a role for Rooibos in the maintenance of normal glucocorticoid levels, which could suggest therapeutic applications for Rooibos in the management of stress-related conditions and metabolic diseases.

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The authors have declared no conflict of interest.

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Supporting Information Table S1: Major phenolic compounds present in aqueous and methanolic extractions of fermented and unfermented Rooibos. Values are expressed as the mean flavonoid content (µg) extracted from 1 g plant material ± SEM.

Flavonoids	Aqueous Rooibos extracts		Methanol Rooibos extracts	
	Fermented	Unfermented	Fermented	Unfermented
Aspalathin	514.8 ± 0.4	21120.0 ± 24.8	927.1 ± 2.4	23560.0 ± 1035.0
Nothofagin	126.4 ± 3.2	3991.0 ± 1.2	248.1 ± 1.8	4915.0 ± 408.7
Rutin	114.0 ± 1.2	614.8 ± 2.0	214.8 ± 0.4	621.9 ± 28.1
Quercetin-3-O-robinobioside	715.2 ± 6.4	1102.0 ± 7.2	1247.0 ± 5.1	1138.0 ± 53.6
Orientin	1013.0 ± 4.0	1392.0 ± 2.8	2656.0 ± 1.4	2356.0 ± 61.2
Isoorientin	1343.0 ± 19.2	2248.0 ± 8.0	3191.0 ± 0.4	3153.0 ± 17.7
Vitexin	217.2 ± 1.2	268.0 ± 1.6	521.8 ± 1.4	435.7 ± 27.6
Isovitexin	222.8 ± 2.8	337.2 ± 1.2	485.2 ± 0.9	477.7 ± 25.7

4.2 Summary

The data obtained in COS-1 cells and in H295R cells clearly shows that Rooibos as well as the flavonoids, aspalathin, nothofagin, orientin, vitexin and rutin interact with and inhibit adrenal steroidogenic enzymes, which subsequently influences the shunt of metabolites through the mineralocorticoid-, glucocorticoid- and adrenal androgen precursor pathways.

In COS-1 cells, Rooibos significantly inhibited the activities of CYP17A1 and CYP21A2, while the inhibitory effect on 3βHSD2 and CYP11B1 was less apparent. The five Rooibos flavonoids, regardless of their structural differences, inhibited the activities of CYP17A1 and 3βHSD2, while inhibition of downstream enzymes, CYP21A2 and CYP11B1 was both flavonoid and substrate specific. The dihydrochalcones, aspalathin and nothofagin, inhibited the activity of CYP21A2 significantly, but not that of CYP11B1. In contrast, the flavones, orientin and vitexin, and the flavonol, rutin, inhibited deoxycortisol conversion by CYP11B1 significantly, while inhibition of DOC conversion by CYP11B1 as well as PROG and 17OH-PROG conversion by CYP21A2 was either negligible or absent. There are thus distinct differences between the inhibitory effects of the dihydrochalcones compared to that of the flavones and the flavanol, which could be attributed to differences in structural flexibility as well as the number and position of their hydroxyl and glucosyl moieties.

In H295R cells, Rooibos significantly reduced total steroid production under both basal and forskolin stimulated conditions. The inhibitory effect was 2-fold greater under stimulated conditions, with Rooibos decreasing end products in the mineralocorticoid-, glucocorticoid- and adrenal androgen precursor pathways. The UPLC-MS/MS method developed for the quantification of steroid metabolites produced by H295R cells enabled the analyses of the steroid flux in the respective pathways. This novel method enabled the separation and quantification of 21 steroid metabolites in the steroidogenic pathways using a single chromatographic separation without prior derivitisation. This method has since proved suitable for the quantification of steroid metabolites both *in vitro* and *in vivo*, enabling analyses of the inhibitory profile of compounds and natural products on adrenal steroid hormone production.

Vitexin had the most significant inhibitory effect on overall steroid production in H295R cells under basal conditions, followed by nothofagin, aspalathin and orientin.

Interestingly, while rutin had no effect under basal conditions, it had the greatest inhibitory effect on total steroid production under forskolin-stimulated conditions, followed by vitexin, nothofagin, aspalathin and orientin. Considering the steroid flux in the steroidogenic pathways, the dihydrochalcones reduced steroid hormone production significantly within the glucocorticoid- and adrenal androgen precursor pathways under both basal and stimulated conditions, while they had no effect on steroid hormone production within the mineralocorticoid pathway. The flavones affected steroid hormone production significantly within the three pathways, and compared to the dihydrochalcones and the flavonol, the flavones were the only compounds that inhibited ALDO production significantly under stimulated conditions. Upon stimulation with forskolin, rutin also reduced steroid production significantly in the three pathways, with the inhibitory effects within the glucocorticoid- and adrenal androgen precursor pathways being significantly greater compared to the inhibition by the other compounds.

In terms of the influence of Rooibos on glucocorticoid production, Rooibos and all the flavonoid compounds investigated reduced glucocorticoid levels significantly under stimulated conditions, but not under basal conditions, with the exception of vitexin. In our analyses of cortisone levels, we detected increased levels of the metabolite in response to forskolin stimulation, while it has been shown to decrease in adrenal primary cultures when cells were stimulated with ACTH. In this study, Rooibos was shown to decrease cortisone significantly under forskolin-stimulated conditions. Reports that the human adrenal expresses both 11βHSD isoforms prompted further investigation into the effects of Rooibos on these enzymes. Rooibos significantly reduced the cortisol:cortisone ratio in CHO-K1 cells expressing 11βHSD1, suggesting that Rooibos favours the dehydrogenase reaction of 11βHSD1. These results were obtained both in the absence and presence of H6PDH, which suggests that Rooibos modulates the activity of 11βHSD1 without interfering with H6PDH. Conversely, Rooibos had no effect on the activity of 11βHSD2.

The two *in vivo* studies further highlighted the influence of Rooibos on adrenal steroidogenesis. The consumption of six cups of Rooibos per day by human subjects at risk for CVD showed that Rooibos significantly influenced glucocorticoid levels as well as steroid metabolite ratios linked to various metabolic diseases. The data obtained in experimental rats administered equivalent doses of Rooibos, underlined

these findings, showing significantly reduced plasma CORT and DOC levels, while also significantly reducing CORT:testosterone ratios. In the human study, the cortisol:cortisone ratio was reduced significantly in both males and females, while cortisone levels were significantly higher in males only. It is interesting to note that testosterone levels were increased in male subjects as well as in male rats receiving Rooibos, while increased DHEA-S levels were detected in female subjects.

Taken together, the data shows that Rooibos affects glucocorticoid biosynthesis either by reducing glucocorticoid biosynthesis by the adrenal and/or via the inhibition of glucocorticoid activation by 11βHSD1. The observation that Rooibos significantly reduced glucocorticoid levels under forskolin stimulated conditions, but not under basal conditions in H295R cells, together with the significant reductions in glucocorticoid levels and ratios *in vivo*, strongly suggests therapeutic applications for Rooibos in the management of stress-related diseases.

CHAPTER 5

General discussion and conclusion

Chronically elevated glucocorticoid levels play a central role in hyperglycemia, T2D, insulin resistance, hypertension, suppression of the immune system, dyslipidemia, visceral obesity and CVDs, which are characteristic of clinical conditions such as MetS and Cushing's syndrome (Chrousos and Gold 1998; Sen et al. 2008; Feelders et al. 2012; Gathercole et al. 2013). Of all the adverse systemic effects associated with elevated glucocorticoid levels, CVDs are the most deleterious and it is currently the leading cause of death globally, with diabetes, insulin resistance, dyslipidemia, obesity and hypertension being major risk factors for the development of CVDs (Arnaldi et al. 2003; Faggiano et al. 2003; Dekkers et al. 2007; Feelders et al. 2012).

Numerous *in vivo* studies have reported that Rooibos consumption significantly improves glucose homeostasis, insulin resistance and lipid profiles, while also exhibiting potent anti-oxidant, anti-diabetic and cardio-protective properties, amongst others (Mose Larsen et al. 2008; Marnewick et al. 2011; Son et al. 2013; Ajuwon et al. 2014; Dludla et al. 2014). In addition, Rooibos has also been reported to modulate the activities of CYP17A1 and CYP21A2, two key enzymes involved in adrenal steroidogenesis (Richfield 2008; Perold 2009). These data suggests that Rooibos may have a functional role within most of the metabolic diseases associated with elevated glucocorticoid levels. The aim of this thesis was therefore to determine the influence of Rooibos on adrenal steroid hormone production *in vitro* and *in vivo*, and in particular, on the levels of the glucocorticoids, cortisol and CORT.

Glucocorticoids are responsible for the regulation of carbohydrate, protein and lipid metabolism - increasing circulating glucose levels at the expense of protein and fat stores. In the liver, glucocorticoids promote gluconeogenesis and glycogen synthesis, while simultaneously stimulating protein catabolism in the muscle and lipolysis in adipose tissue to fuel gluconeogenesis. Furthermore, glucocorticoids also reduce glucose uptake and insulin sensitivity in tissues (Costanzo 2014). In response to the high blood glucose levels caused by the actions of glucocorticoids, insulin is secreted from pancreatic β-cells to promote glucose uptake, glycogenesis and lipogenesis, while lipolysis is inhibited. Patients with T2D, however, are insulin

resistant and circulating glucose levels remain chronically elevated, which results in a wide range of pathological consequences. Treatment of T2D include drugs that target glucose-regulating processes in the pancreas, liver, skeletal muscle, gut or adipose tissue (Mohler et al. 2009). Rooibos was recently patented as an anti-diabetic agent due to the ability of Rooibos extract and two major Rooibos flavonoid compounds, aspalathin and rutin, to significantly reduce plasma glucose levels and improve impaired glucose tolerance *in vivo* (Mose Larsen et al. 2008; Kawano et al. 2009; Muller et al. 2012; Son et al. 2013). In addition, aspalathin was shown to suppress the expression of genes encoding enzymes involved in gluconeogenesis, glycogenolysis and lipogenesis *in vivo*, while also increasing glucose uptake in muscle cells and promoting insulin secretion from pancreatic β-cells *in vitro* (Kawano et al. 2009; Son et al. 2013).

Various studies have reported that diabetes as well as CVDs are linked to oxidative stress, with the severity of diabetes being positively correlated to the formation of ROS (Brownlee 2005; Halliwell 2006; Houstis et al. 2006; Valko et al. 2006). Rooibos, which is well known for its potent anti-oxidant properties, has been reported to reduce oxidative stress *in vivo* by counteracting the oxidative stress-induced reduction in GSH:GSSH ratios, while also significantly reducing lipid-peroxidation and pro-inflammatory cytokine secretion (Marnewick et al. 2003; Ajuwon et al. 2014; Hong et al. 2014). Macrophages that accumulate in adipose tissue are a major source of the pro-inflammatory cytokines, IL-6 and TNF-α. Inhibition of TNF-α production, which has been reported to be elevated in the blood and adipose tissue of obese animals, was shown to significantly improve insulin sensitivity (Hotamisligil 1999; Shoelson et al. 2006). Furthermore, TNF-α and IL-6 released by adipocytes and macrophages are elevated in diabetic and insulin resistant patients (Senn et al. 2002). The crosstalk between inflammation and ROS in diabetes is also evident, since ROS has been shown to promote the production of TNF-α and IL-6 (Rahman et al. 2002). It is therefore likely that agents capable of suppressing inflammation by reducing pro-inflammatory cytokine levels and/or their biological actions may have beneficial effects in the treatment of insulin resistance and/or diabetes (Kim et al. 2004; Larsen et al. 2007; Sears and Ricordi 2012; Gómez-Zorita et al. 2013; Siriwardhana et al. 2013). Rooibos extract has been reported to exhibit potent anti-inflammatory effects both *in vitro* (Hendricks and Pool 2010; Mueller et al. 2010) and *in vivo* (Baba et al. 2009) and preliminary results from our group have shown that

Rooibos decreased IL-6 production in rat adrenal tissue, while significantly increasing the levels of the anti-inflammatory cytokine, IL-10 ($P<0.05$) (unpublished data). It is therefore possible that the anti-diabetic properties of Rooibos could also be ascribed to its anti-inflammatory properties.

As mentioned previously, hypertension as well as the aforementioned metabolic diseases are major risk factors for the development of CVDs (Arnaldi et al. 2003; Faggiano et al. 2003; Dekkers et al. 2007; Feelders et al. 2012). Due to the strong association between the RAAS and blood pressure regulation, blockade of this system is a key therapeutic target for the treatment of hypertension, with ACE inhibitors currently being one of the most clinically relevant pharmacological agents for the treatment of hypertension and CVD (Persson et al. 2010; van Vark et al. 2012). In a human study by Persson et al. (2010), a single oral dose of Rooibos was shown to significantly inhibit the activity of ACE at 30 min and 60 min after intake (Persson et al. 2010). It was subsequently shown that Rooibos exhibited its inhibitory action of ACE via the same mechanism as enalaprilat, a known ACE inhibitor (Persson 2012).

In addition to hypertension, dyslipidemia, another major risk factor for CVD, is characterized by elevated plasma total cholesterol, LDL cholesterol and TGs and reduced levels of HDL cholesterol. In a study by Francisco (2010), a single oral dose of Rooibos significantly reduced total cholesterol, LDL cholesterol and TGs as well as inflammatory biomarkers and plasma glucose and insulin levels in normolipidemic subjects (Francisco 2010). These findings were corroborated in a subsequent study by Marnewick et al. (2011), reporting that Rooibos consumption significantly decreased plasma LDL cholesterol and TGs, while increasing the levels of HDL cholesterol in humans at risk for CVD (Marnewick et al. 2011). These findings suggest Rooibos to have positive modulatory effects in normal individuals as well as in disease states. In addition, Rooibos was shown to exhibit similar beneficial effects in a hyperlipidemic mouse model, as reflected in significant reductions in serum cholesterol, TG and FFA levels. Rooibos also protected the liver from lipid storage and prevented the accumulation of fat in adipocytes as well as the development of dietary-induced hepatic steatosis (Beltrán-Debón et al. 2011).

Taken together, these data clearly demonstrate a role for Rooibos in the treatment and perhaps prevention of numerous metabolic disorders such as T2D, insulin resistance, hypertension, dyslipidemia and obesity, which are all major risk

factors for CVDs. Since these conditions are strongly linked with chronically elevated glucocorticoid levels, this study investigated whether Rooibos can reduce glucocorticoid levels through inhibiting the activities of steroidogenic enzymes involved in glucocorticoid homeostasis.

The final step of glucocorticoid biosynthesis in the adrenal is catalysed by CYP11B1. However, glucocorticoid levels are not only dependent on the activity of CYP11B1, but also on the activities of upstream enzymes such as CYP21A2, 3βHSD2 and CYP17A1, which catalyse a network of reactions to yield substrates for CYP11B1. Previous studies in our laboratory by Richfield (2008) and Perold (2009) showed that Rooibos significantly inhibited the activities of CYP17A1 and CYP21A2, expressed in COS-1 cells. (Richfield 2008; Perold 2009). In the present study, these investigations were continued by analysing the influence of Rooibos extract and five major Rooibos flavonoids (the dihydrochalcones, aspalathin and nothofagin, their flavone analogues, orientin and vitexin and the flavonol, rutin) on the activities of CYP17A1, 3βHSD2, CYP21A2 and CYP11B1, expressed in COS-1 cells. The results in the present study corroborated previous results by Richfield (2008) and Perold (2009), with Rooibos significantly inhibiting the catalytic activities of both CYP17A1 and CYP21A2 in COS-1 cells, while the inhibitory effects on 3βHSD2 and CYP11B1 were negligible. 3βHSD2 and CYP17A1 were inhibited in the presence of all the flavonoid compounds assayed, while inhibition of the downstream enzymes, CYP21A2 and CYP11B1 was both flavonoid and substrate specific. The dihydrochalcones inhibited the activity of CYP21A2 significantly, but not that of CYP11B1. In contrast, the flavones and the flavonol inhibited deoxycortisol conversion by CYP11B1 significantly, while inhibition of PROG and 17OH-PROG conversion by CYP21A2 as well as DOC conversion by CYP11B1 was either negligible or absent. From these results it is clear that there are distinct differences between the dihydrochalcones, flavones and the flavonol in terms of their inhibitory effects on adrenal steroidogenesis, which could be attributed to the structural differences of these flavonoid compounds in terms of the number and position of their hydroxyl and glucose moieties, as well as differences in the structural flexibility of these compounds. These data are in agreement with previous results by Ohno et al. (2002), showing that the flavonoids, daidzein, genistein and 6-hydroxyflavone selectively inhibited the activities of steroidogenic enzymes in H295R cells (Ohno et al. 2002).

After having studied the effects of Rooibos in isolated cell model systems, the influence of Rooibos and selected flavonoids were investigated on steroid hormone production in human adrenal H295R cells, under both basal and forskolin stimulated conditions, mimicking the effects of ACTH which increases glucocorticoid production in response to stress. In a recent study by Xing et al. (2011), the influence of ACTH on steroid production was determined in adult adrenal primary cultures. ACTH treatment increased the levels of the glucocorticoids, cortisol (63-fold), CORT (37-fold), the glucocorticoid precursor, deoxycortisol (23-fold), as well as the adrenal androgen precursors, A4 (26-fold), DHEA (18-fold) and 11OHA4 (17-fold) significantly. Cortisol represented 30% of the total steroids detected in under basal conditions and 61% following ACTH treatment, confirming the ability of ACTH to stimulate glucocorticoid biosynthesis under the control of the HPA-axis. The authors continued their investigations in H295R cells, using forskolin instead of ACTH as an agonist. In terms of the total steroids detected in H295R cells, cortisol represented 10% under basal conditions and 26% following forskolin stimulation (Xing et al. 2011).

In our analyses of steroid production by H295R cells, comparable results were obtained, with cortisol comprising 15% of the total steroids detected under basal conditions and 34% following stimulation with forskolin, confirming the ability of forskolin to increase cortisol production, mimicking the stress response *in vitro*. Under basal conditions, Rooibos had no effect on the levels of cortisol. However, upon forskolin stimulation, Rooibos significantly limited the forskolin-induced increase in cortisol levels (5-fold). With the exception of vitexin, which reduced basal cortisol levels 1.3-fold, none of the other flavonoids inhibited cortisol production under basal conditions. Under stimulated conditions, however, all the flavonoids significantly reduced cortisol levels between 1.3- and 3-fold when compared to untreated controls. Of all the flavonoids assayed, Rutin had the greatest inhibitory effect on cortisol biosynthesis, as it reduced stimulated cortisol levels from 3.8 µM to 1.2 µM, although these levels were still 2-fold higher than that of basal cortisol levels (0.7 µM). The 5-fold reduction observed in the presence of Rooibos extract, on the other hand, reduced the levels of cortisol from 3.8 µM to 0.8 µM, similar to that of basal cortisol levels. Despite their varying inhibitory capacities, all the flavonoid compounds significantly reduced the levels of cortisol under stimulated conditions, although not to the same extent as the Rooibos extract. The reduction in cortisol levels by Rooibos

was, however, not due to inhibition of CYP11B1, but rather due to upstream inhibition of CYP17A1 and CYP21A1, resulting in decreased delivery of substrate to CYP11B1.

Within the mineralocorticoid pathway, the levels of ALDO were unaffected under basal conditions, however, in response to forskolin treatment, the flavones, orientin and vitexin limited the elevation in ALDO levels significantly. In addition, the levels of the mineralocorticoid precursors were significantly lower in the presence of Rooibos and rutin under stimulated conditions. It is possible that, depending on levels of these compounds in Rooibos extracts and the degree of absorption in the gut, that these flavones may impact hypertension and CVD, together with the inhibition of ACE as previously reported (Persson et al. 2010; Persson 2012). It should, however, be noted that the levels of ALDO comprised 0.1% of the total steroids detected under basal conditions and 0.2% under forskolin-stimulated conditions. Stimulating the H295R cells with Ang II or KCl to increase the levels of ALDO would perhaps present a more appropriate model for an investigation into the effect of Rooibos on the mineralocorticoid pathway, allowing more definite conclusions to be drawn.

In the adrenal androgen pathway, the levels of testosterone, as well as its precursor metabolite, A4, was significantly inhibited by Rooibos and all the flavonoid compounds assayed under both basal and forskolin stimulated conditions, with the exception of rutin, which did not have any inhibitory effect on steroid biosynthesis under basal conditions. Rooibos significantly reduced the steroid flux through the adrenal androgen precursor pathway, as is reflected in the increased levels of PREG. These results, suggesting inhibition of CYP17A1, were confirmed in COS-1 cells, as Rooibos reduced the activity of CYP17A1 by ±50%. In the human study, the levels of testosterone and DHEA-S, as well as the cortisol:testosterone and cortisol:DHEA-S ratios were unaffected following Rooibos consumption. These results were also reflected in rats, with testosterone levels not being affected by Rooibos.

While Rooibos had negligible effect on circulating cortisol levels, Rooibos significantly reduced the cortisol:cortisone ratio (P=0.0486) in the plasma of both male and female subjects at risk for CVD. In addition, Rooibos also increased plasma cortisone levels in male subjects significantly (P=0.0465). In male Wistar rats, Rooibos reduced the CORT:11-DHC ratio to a level approaching significance (p=0.0624), with the levels of CORT (P=0.0275), and its precursor metabolite, DOC (P=0.0298), being significantly reduced following Rooibos consumption. In addition, Rooibos significantly reduced the CORT:testosterone ratio (P=0.0009) which, if

elevated, has been linked to the development of CVD (Rosmond et al. 2003; Smith et al. 2005).

Since Rooibos did not inhibit the activity of CYP11B1, it is possible that the reduced cortisol:cortisone ratios observed in the human study could be attributed to altered activity of 11βHSD1 and/or 11βHSD2, since these isozymes catalyse the inter-conversion between cortisol and cortisone. In a recent study by Harno et al. (2013), it was proposed that intracellular glucocorticoids regenerated in the liver via 11βHSD1, rather than circulating glucocorticoids, contribute towards the development of MetS, and that selective inhibition of 11βHSD1 seems to be a promising target for the treatment of MetS (Harno et al. 2013). Selective inhibition of 11βHSD1 has been reported to prevent hyperglycemia, dyslipidemia and obesity, while also improving glucose homeostasis, lipid profiles, glucose tolerance and insulin tolerance (Masuzaki et al. 2003; Stewart 2003; Cooper and Stewart 2009; Anagnostis et al. 2013). Selective inhibition of 11βHSD1 is, however, crucial, since inhibition of 11βHSD2 results in inappropriate cortisol binding to the MRs in the kidney, which leads to “apparent mineralocorticoid excess” syndrome, hypertension, hypokalemia and suppression of the RAAS (Russo et al. 2000; Anagnostis et al. 2010, 2013; Pant et al. 2010).

In the present study it was demonstrated that Rooibos significantly inhibited the activity of 11βHSD1 expressed in CHO-K1 cells, in the absence and presence of H6PDH, as reflected in the significant reduction in the cortisol:cortisone ratio ($P<0.01$), while no effect on the cortisol:cortisone ratio was observed in cells expressing 11βHSD2.

Taken together, the data presented in this thesis clearly shows that Rooibos and major Rooibos flavonoid compounds significantly inhibited the activities of key adrenal steroidogenic enzymes *in vitro*, which subsequently altered the steroid flux through the mineralocorticoid-, glucocorticoid- and adrenal androgen precursor pathways. The observation that Rooibos did not affect cortisol production under basal conditions in H295R cells, while significantly reducing elevated cortisol levels in response to forskolin treatment to levels characteristic of the basal milieu, strongly suggests a therapeutic role for Rooibos in the management of clinical conditions associated with elevated glucocorticoid levels. The significant reductions in cortisol levels appeared to be due to inhibition of upstream enzymes rather than CYP11B1. Rooibos did, however, significantly inhibit the activity of 11βHSD1 *in vitro*, as is

evident in the considerable reduction in the cortisol:cortisone ratios, while the activity of 11βHSD2 remained unaffected. These *in vitro* results were also reflected *in vivo*, as Rooibos significantly reduced the cortisol:cortisone ratios in human subjects at risk for CVD. While the reduction in the CORT:11-DHC ratio in rats only approached significance, Rooibos significantly reduced the levels of the glucocorticoid, CORT, and its precursor metabolite, DOC. It therefore appears that Rooibos affects glucocorticoid biosynthesis in one of two ways – either by reducing glucocorticoid levels in the adrenal via inhibition of upstream enzymes, or by favouring the inactivation of cortisol by 11βHSD1 in peripheral tissues. The data presented in this thesis, along with previous studies reporting on the anti-oxidant, anti-diabetic and cardio-protective properties of Rooibos, suggests a therapeutic role for Rooibos as a functional food to aid in the treatment of metabolic diseases associated with elevated glucocorticoid levels.

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ADDENDUM A

Table 1: Major flavonoid compounds (µg) present in the methanolic extract of unfermented Rooibos plant material and in the extract administered to COS-1 cells and H295R cells in the present study.

Flavonoid compounds	Rooibos [a]	COS-1 cells [b]	H295R cells [c]
Dihydrochalcones			
Aspalathin	23562.0 ± 1035.0	641.1 ± 28.2	149.1 ± 6.5
Nothofagin	4915.0 ± 408.7	133.8 ± 11.1	31.1 ± 2.6
Flavones			
Orientin	2356.0 ± 61.2	64.1 ± 1.7	14.9 ± 0.4
Iso-orientin	3153.0 ± 17.7	85.9 ± 0.5	20.0 ± 0.1
Vitexin	435.7 ± 27.6	11.9 ± 0.8	2.8 ± 0.2
Iso-vitexin	477.7 ± 25.7	13.0 ± 0.7	3.0 ± 0.2
Luteolin-7-*O*-glucoside	144.1 ± 14.8	3.9 ± 0.4	0.9 ± 0.1
Flavonols			
Rutin	621.9 ± 28.1	16.9 ± 0.8	3.9 ± 0.2
Quercetin-3-*O*-robinobioside	1138.0 ± 53.3	31.0 ± 1.5	7.2 ± 0.3
Iso-quercetrin	360.5 ± 20.6	9.8 ± 0.6	2.3 ± 0.1
Hyperoside	319.3 ± 13.4	8.7 ± 0.4	2.0 ± 0.1

Values are expressed as the mean ± SEM.

[a] Flavonoids extracted from 1g unfermented Rooibos plant material (methanolic extract).

[b] Flavonoids present in Rooibos extract administered to COS-1 cells / ml media.

[c] Flavonoids present in Rooibos extract administered to H295R cells / ml media.