The influence of *Sutherlandia frutescens* on adrenal steroid hormones and downstream receptor interactions.

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Dedication

This thesis is dedicated to Caitlyn and Sebastian.

"You have brains in your head.
You have feet in your shoes.
You can steer yourself
Any direction you choose."
-Dr Seuss

"The more you read, the more things you will know.
The more you learn, the more places you'll go!"
-Dr Seuss

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Declaration

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Abstract

The study was aimed at:

1. linking anti-stress properties of *Sutherlandia frutescens* to its influence on adrenal steroid biosynthesis in terms of extracts, sutherlandioside (SU) compounds and gamma-aminobutyric acid (GABA).
2. linking anti-inflammatory and anti-hypertension properties to steroid receptor interaction, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR).

The influence of *S. frutescens* extracts, and SUs, on key steroidogenic enzymes was assayed in COS-1 cells and binding to adrenal P450-dependent enzymes was assayed in ovine adrenal mitochondrial and microsomal suspensions. The effects of the methanol extract and sutherlandioside B (SUB) were assayed on basal, forskolin and angiotensin II (Ang II) stimulated hormone levels in the adrenal H295R cell model. The effect of GABA on basal steroid production was investigated in H295R cells. Agonist activity of the methanolic extract and SUB, for both transactivation and transrepression of GR and MR mediated gene transcription, was subsequently investigated.

S. frutescens extracts affected all the steroidogenic enzymes with inhibition being substrate specific. SUB had a greater effect on substrate binding to mitochondrial and microsomal P450 enzymes than sutherlandioside A (SUA). SUB inhibited the catalytic activity of CYP17A1, CYP11B2 and 3β-HSD2 while not affecting CYP21A2. In H295R cells, while the extract decreased total steroid production under basal and forskolin stimulated conditions, only cortisol and its precursor, deoxycortisol, and mineralocorticoid metabolites were decreased significantly in the presence of forskolin. A SU mixture did not influence steroid production, with only cortisol levels decreasing significantly with SUB decreasing cortisol, deoxycortisol, androstendione and 11-hydroxyandrostenedione significantly. The data show that the SU mixture was not able to elicit the same effects as SUB, suggesting that the compounds do not act synergistically. GABA significantly decreased adrenal steroid production in H295R cells with the greatest inhibitory effect observed in the production of adrenal androgens.

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S. frutescens extracts and SUB repressed NF-κB-driven gene expression without activating GRE-driven gene expression while neither activated MR mediated gene transcription while both antagonized the effects of aldosterone via the MR.

The results of this study provide evidence linking anti-stress, anti-inflammatory and anti-hypertensive properties of *S. frutescens* to the inhibition of steroidogenic enzymes and modulation of adrenal hormone biosynthesis. The findings suggesting that *S. frutescens* and SUB exhibit dissociated glucocorticoid characteristics underline potential therapeutic applications in the treatment of inflammation and hypertension.

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Opsomming

Die studie het ten doel gehad om:

1. die koppeling van die anti-stres eienskappe van ekstrakte van *Sutherlandia frutescens*, die sutherlandiosiede (SU) en gamma-aminobottersuur (GABA) aan die plant se invloed op adrenale steroïedbiosintese.
2. die koppeling van anti-inflammatoriese- en anti-hipertensie-eienskappe aan steroïed-reseptor interaksie in terme van die glukokortikoïedreseptor (GR) en die mineralokortikoïedreseptor (MR).

Die invloed van *S. frutescens* ekstrakte en SU op sleutel steroïedogenise ensieme is in COS-1-selle ondersoek. Die binding aan adrenale P450-afhanklike ensieme is ook ondersoek deur gebruik te maak van skaapbynier mitochondriale en -mikrosomale suspensies. Die invloed van ‘n metanol ekstrak en sutherlandiosied B (SUB) is op basale, forskolien en angiotensien II (Ang II) gestimuleerde hormoonvlakke in die H295R-bynierselmodel geassesseer. Die effek van GABA op basale steroïedproduksie in H295R-selle is ook ondersoek. Agonistiese aktiwiteit van die metanolekstrak en SUB, vir beide transaktivering en transrepresie van GR- en MR-gemedieerde geen transkripsie, is vervolgens ondersoek.

S. frutescens ekstrakte het al die steroidogeniese ensieme beïnvloed en het substraatspesifieke inhibisie getoon. SUB het 'n groter effek as sutherlandioside A (SUA) op substraatbinding aan mitochondriale- en mikrosomale P450-afhanklike ensieme gehad. SUB inhibeer die katalitiese aktiwiteit van CYP17A1, CYP11B2 en 3β-HSD2 terwyl CYP21A2 nie deur hierdie verbinding ge-affekteer word nie. Die ekstrak het totale steroïedproduksie onder basale en forskolien-gestimuleerde toestande in H295R-selle verlaag. Slegs kortisol-, sy voorloper deoksikortisol- en mineralokortikoïedmetabolietvlakke is egter beduidend verlaag in die teenwoordigheid van ekstrakte. Steroïedproduksie is nie deur 'n SU-mengsel beïnvloed nie en slegs kortisolvlakke het beduidend afgeneem. Daarenteen het SUB kortisol-, deoksikortisol-, androsteendioon en 11-hidroksi-androsteendioon vlakke beïnvloed. Die data toon dat die SU mengsel nie dieselfde effekte as SUB kon ontlok nie, wat daarop dui dat die verbindings nie sinergisties optree nie. GABA het 'n aansienlik afname in byniersteroïedproduksie in H295R-selle tot gevolg gehad, met die grootste inhibitoreise effek op die produksie van adrenale androgene.

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S. frutescens ekstrakte en SUB het NF-κB-gedrewe geenekspressie onderdruk sonder om GRE-gedrewe geenekspressie te aktiveer maar geen invloed getoon op die MR gemedieerde geentranskripsie getoon nie. Beide die ekstrak en SUB het as aldosteroon antagoniste via die MR opgetree.

Die resultate van hierdie studie bied bewyse wat die anti-stres-, anti-inflammatoriese en anti-hipertensiewe eienskappe van *S. frutescens* verbind met die inhibisie van adrenale steroïedogeniese ensieme en gevolglike modulering van adrenale hormoonbiosintese. Die bevindinge dui duidelik daarop dat *S. frutescens* en SUB dissiatiewe glukokortikoïedeienskappe vertoon, en wys op moontlike terapeutiese toepassings in die behandeling van inflammasie en hipertensie.

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List of Abbreviations

11-DHC	Dehydrocorticosterone
11OHA4	11-hydroxyandrostenedione
11OHT	11β-hydroxytestosterone
16OH-PROG	16 hydroxy-progesterone
17OH-PREG	17 hydroxy-pregnenelone
17OH-PROG	17 hydroxy-progesterone
18OH-CORT	18-hydroxycorticosterone
3α,5α-THDOC	3α,5α-tetrahydrodeoxycorticosterone
3α,5α-THPROG	3α,5α-tetrahydroprogesterone
3β-HSD2	3β-hydroxysteroid dehydrogenase type 2
5α-DHDOC	5α-dihydrodeoxycorticosterone
5α-DHPROG	5α-dihydroprogesterone
A4	Androstenedione
ACE	angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
AF-1	Transcriptional activation function 1
AF-2	Transcriptional activation function 2
AIDS	Acquired immunodeficiency syndrome
ALDO	Aldosterone
AngI	Angiotensin I
AngII	Angiotensin II
ANS	Autonomic nervous system
AP-1	Activator protein-1

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APS	Adenosine phosphosulfate
AR	Androgen receptor
AT-1 receptor	Angiotensin II receptor Type 1
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BMAL1	Brain muscle ARNT-like 1
cAMP	Cyclic adenosine monophosphate
CASPs	Caspases
CBG	Corticosteroid-binding globulin
CHO	Chinese Hamster Ovarian
CLOCK	Circadian locomotor output cycles kaput
COPD	Chronic obstructive pulmonary disease
CORT	Corticosterone
COX	Cyclooxygenase
CRH	Corticotrophin release hormone
CYP	Cytochrome P450
CYP11A1	Cytochrome P450 side-chain cleavage
CYP11B1	Cytochrome P450 11β-hydroxylase
CYP11B2	Aldosterone synthase
CYP17A1	Cytochrome P450 17α-hydroxylase/17,20-lyase
CYP21A2	Cytochrome P450 21-hydroxylase
DBD	DNA-binding domain
Dex	Dexamethasone
DHEA	Dehydroepiandrosterone

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DHEAS	Dehydroepiandrosterone sulfate
DMEM	Dulbecco's Modified Eagle's medium
DOC	Deoxycorticosterone
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ER	Estrogen receptor
ESI	Electron spray ionization
FAD	Flavin adenine dinucleotide
FASLG	FAS ligand
Fedx	Ferrodoxin
FeRed	Ferrodoxin reductase
FKBP5	FK506 binding protein
FMN	Flavin mononucleotide
GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase
GI	Gastro-intestinal
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
HIV	human immunodeficiency virus
HPA-axis	Hypothalamic-pituitary-adrenal-axis
HPLC	High pressure liquid chromatography
HSD	Hydroxysteroid dehydrogenase
IFN-γ	Interferon gamma
IL-6	Interleukin-6

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JNK	Jun amino-terminal kinases
LBD	Ligand-binding domain
LC-MS	Liquid chromatography-mass spectrometry
LC–MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LPS	Lipopolysaccharides
MAPK	mitogen-activated protein kinases
MCR2	Melanocortin type 2 receptor
MR	Mineralocorticoid receptor
MRE	Mineralocorticoid response element
MTT	3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide
Na+,K+-ATPase	Sodium-potassium adenosine triphosphatase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NF-κB	Nuclear factor kappa B
NL	Nuclear localization
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
P450	Cytochrome P450
PAPS	3’-phosphoadenosine-5’-phosphosulfate
PASS1	PAP synthase type 1
PASS2	PAP synthase type 2
PBS	Phosphate buffered saline
p-ERK	Phosphorylated-extracellular signal-regulated kinase

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PI3-K	phosphoinositide 3-kinase
PKA	Protein kinase A
PMA	Phorbol 12-myristate 13-acetate
POMC	Proopiomelanocortin
POR	P450 oxidoreductase
PPT	Protopanaxatriol
PR	Progesterone receptor
PREG	Pregnenolone
PREGS	PREG sulfate
PROG	Progesterone
PVN	Paraventricular nucleus
RCL	Reactive center loop
ROS	Reactive oxygen species
RT	Reverse transcriptase
SCN	Suprachiasmatic nucleus
SEDIGRAM	Selective dimerizing glucocorticoid receptor agonists or modulator
SEGRA	Selective glucocorticoid receptor agonist
SEGRAM	Selective glucocorticoid receptor agonist or modulators
SEMOGRAM	Selective monomerizing glucocorticoid receptor agonists and modulator
SHBG	Sex hormone binding globulin
SNS	Sympathetic nervous system
SPL	Spironolactone
SRS	Substrate recognition sites
StAR	Steroidogenic acute regulatory protein

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STAT3	Signal transducer and activator of transcription 3
SU	Sutherlandioside
SULT	Sulfotransferase
TIC	Total ion chromatogram
TPA	12-O-tetradecanoylphorbol-13-acetate
UPLC–MS/MS	Ultra performance liquid chromatography coupled with tandem mass spectrometry
V1b	Vasopressin 1b

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Chapter 1: Introduction

Recent commercial interest in herbal therapies has resulted in increased scientific research into the validity, efficacy and safety of herbal medicines in South Africa. The potential integration of complementary and alternative medicines into conventional medicinal practices in South Africa has also contributed to increased scientific interest in herbal therapeutics (van Wyk, 2011). Although initial studies focussed on the effects of whole plant extracts, the identification of specific compounds within extracts has been of particular interest in developing medicines. Chemical profiling of different solvent extracts has resulted in the identification of specific compounds, many of which have been associated with specific therapeutic applications. *Sutherlandia. frutescens*, a common southern African herbal remedy, has been traditionally used to treat a variety of ailments including stress, anxiety, inflammation and cancers (van Wyk, 2008). This study investigated the anecdotal claims of the use of *S. frutescens* in the treatment of stress, anxiety, depression and inflammation by investigating the effects of extracts and compounds on adrenal steroidogenesis and downstream steroid receptor interactions, specifically the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), respectively.

Chapter 2 describes the anecdotal therapeutic claims associated with *S. frutescens* −studies that have confirmed therapeutic applications of the extracts and the compounds identified within the extract that may elicit biological effects. For the purposes of this study, studies describing the effects of *S. frutescens* on inflammation, cancers, stress and anxiety as well as immunodeficiency disorders were highlighted. In addition, compounds that may be responsible for eliciting the aforementioned effects were addressed with particular focus on amino acids, flavonoids and triterpenoids. The triterpenoids were deemed especially important in the context of this study as these compounds share structural similarities with adrenal steroid hormones. As therapeutic effects cannot be elicited without compounds within the extract reaching the appropriate biological target, the manner in which structure-related characteristics may influence the bioavailability of the compounds were addressed. Recent reports regarding safety and efficacy aspects of the use of *S. frutescens* were included in the overview.

Previous studies have shown that *S. frutescens* extracts may interact with adrenal cytochrome P450 (P450) enzymes (Prevoo et al., 2008), potentially resulting in the reduction of corticosteroid production. In Chapter 3, the regulation, biosynthesis, peripheral actions and transport mechanisms of corticosteroids with particular focus on adrenal steroidogenesis, the

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structure and function of these steroids and the adrenal steroidogenic enzymes that catalyse their biosynthesis are discussed. In addition, upstream regulation, mainly via the hypothalamic-pituitary-adrenal (HPA)-axis, and the downstream effects of corticosteroids through interactions with the GR and MR were also included.

We hypothesized that the anecdotal anti-inflammatory effects of *S. frutescens* could be attributed to the influence of the extract on adrenal steroidogenesis and the downstream activation of steroid receptors. We further hypothesized that the presence of compounds, such as the sutherlandioside (SU) compounds and gamma-aminobutyric acid (GABA), in *S. frutescens* may contribute to anecdotal effects. In this study, we thus aimed to:

- Determine the effect of a methanolic extract of *S. frutescens* on single adrenal steroidogenic enzymes and on steroid production in the H295R adrenal cell model.
- Quantify the SU and GABA content of the extract used in this study.
- Determine the effects of SUs on single adrenal steroidogenic enzymes and *in vitro* steroid production.
- Determine the downstream effects of the methanolic *S. frutescens* extract and SUB by investigating their transcriptional activities via the GR and MR.
- Determine the effects of GABA on adrenal steroid production in H295R cells.

Chapter 4 addresses the main hypothesis of the study and the *in vitro* effects of a methanolic *S.frutescens* extract on adrenal steroidogenesis as well as GR and MR mediated transcription. The presence of SUB and GABA within these extracts was confirmed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) and the effects of these compounds on adrenal steroidogenesis was investigated. Findings pertaining to the potential anti-inflammatory, anti-stress and anti-hypertensive applications of the extract are also described in Chapter 4. Results contained in Chapter 4 have been published in part (Sergeant et al., 2017) and the publication has been added to this thesis as Addendum I. In addition to the main hypothesis, it was hypothesized that GABA may influence adrenal steroid production at the site of production in the adrenal gland and subsequently investigated the influence of GABA on steroid production by P450 enzymes in H295R cells. These results are presented and discussed in Chapter 5.

Chapter 6 concludes this body of work and describes the potential implications of the novel findings reported in this study. Findings are summarised to conclude the thesis −the anti-

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inflammatory and anti-hypertensive effects of the extract, as well the motivation for the use of *S. frutescens* as a supplementary aid in anti-inflammatory and anti-stress treatments.

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Chapter 2: *Sutherlandia frutescens* as a medicinal plant

Introduction

S. frutescens, also known as *Lessertia frutescens* and cancer bush, has been used in southern Africa for the treatment of a wide spectrum of ailments, many without scientific validation. Some of the first records of the medicinal use of decoctions made from the aerial parts of these plants date back to the Khoi San and Nama people, who used *S. frutescens* to treat fevers and to wash wounds. Even the present day inhabitants of the Western Cape still commonly use *S. frutescens* as a traditional herbal medicine (van Wyk, 2008).

Therapeutic applications

The use of *S. frutescens* as herbal remedy has been mostly associated with traditional use and the distribution of traditional *S. frutescens* derived medicines still largely depends on traditional healers, herbalists and so-called bush doctors. After harvesting fresh *S. frutescens* leaves, pods, flowers and/or stems, the parts are dried, mashed into a fine powder and served as an infusion. Traditional healers are known to prepare the *S. frutescens* infusions according to the type and severity of disease as indicated by the patient (van Wyk and Albrecht, 2008). Traditionally the aerial parts of the plant are used to reduce fevers and wash wounds, whereas teas made from the leaves and stems are used to treat cancer, fever, stomach ailments, diabetes, depression and inflammation (Thring and Weitz, 2006; van Wyk and Wink, 2004). Although *S. frutescens* is most commonly associated with traditional herbal medicines, tablets, gels, capsules and ointments have been produced and are available commercially (van Wyk, 2008; van Wyk, 2011). Limited local commercialization of *S. frutescens* products has occurred with limited scientific validation, however there have been studies to scientifically appraise its medicinal value. These studies, specifically those relevant to this thesis, will be discussed in the sections below.

Inflammation

The treatment of chronic inflammation with herbal medicines has been a topic of investigation for many years (Iwalewa et al., 2002). Traditional healers often use a single plant to treat a wide range of diseases and there is often a common target for all these ailments such as the inflammatory response. Although the inflammatory response is considered as a whole, specific parameters, such as cytokine production, glucocorticoid production and the transcriptional activities of steroid receptors have been identified as potential targets for anti-inflammatory treatment. In addition, chronic inflammation appears to be an underlying issue in many disease states, including chronic heart failure, hypertension, diabetes and cancers (Kotas and

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Medzhitov, 2015). Scientific research into the effect of *S. frutescens* on the inflammatory response and the application thereof as an anti-inflammatory treatment far outweighs the study of this botanical as a treatment for other disease states.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used to treat inflammation since the early 1970s (Whitehouse, 2011). Despite the wide application of NSAIDs in the treatment of inflammation, the chronic use of these drugs is usually associated with negative side effects that have been attributed to these drugs non-selectively inhibiting both cyclooxygenase (COX)-1 and 2 (Brune and Patrignani, 2015). The therapeutic use of NSAIDs in the treatment of inflammation arises from the inhibition of the inducible COX-2 enzyme in inflamed tissues. COX-1, however, is constitutively expressed in tissues such as the kidney and stomach and is responsible for the biosynthesis of prostaglandins that are engaged in homeostasis. The inhibition of COX1 contributes to the adverse effects associated with the use of NSAIDs (Seibert et al., 1994). The ability of drugs or compounds to specifically inhibit COX-2 has therefore been of particular interest in finding anti-inflammatory treatments. Kundu et al. (2005) showed that methanolic extracts of *S. frutescens* was able to inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 expression in mouse skin when applied topically (Kundu et al., 2005). Similarly, Na et al. (2004) showed that COX-2 activity, as well as its induction by nuclear factor kappa B (NF-κB), are significantly inhibited by the presence of *S. frutescens* extracts (Na et al., 2004). Further investigations into the anti-inflammatory potential of *S. frutescens* extracts revealed that the extracts have the ability to significantly reduce inflammation in albumin induced paw edema in mice. In the same study, the use of *S. frutescens* extracts in pain relief was investigated. Hot plate and acetic acid models were used to induce pain and the effects of *S. frutescens* extracts (500-800 mg/kg) were compared to the analgesic effects of diclofenac (100 mg/kg) and chloropropamide (250 mg/kg). The *S. frutescens* root extract was shown to significantly reduced thermally and chemically induced nociceptive pain stimuli in mice (Ojewole, 2004).

Tissue damage at the site of inflammation is another negative side effect that can potentially result from chronic inflammation. The accumulation of reactive oxygen species (ROS) during the inflammatory response as a result of increased inflammatory mediators has been linked to tissue damage due to oxidative stress. Increased oxygen uptake, caused by the increase in inflammatory mediators, and the consequential increased metabolism of molecular oxygen causes the accumulation of ROS. Antioxidants inactivate ROS through conversion to less harmful compounds that can easily be eliminated from the body. Cell damage or cell death may

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occur due to the resulting oxidative stress when there is an imbalance between ROS and antioxidants in the favour of ROS, (Fernández-Sánchez et al., 2011). The role of antioxidants in the protection of tissues during chronic inflammation should thus not be disregarded.

Although there have been studies to show that *S. frutescens* extracts display no antioxidant potential, several other studies have confirmed that extracts from these plants do display antioxidant activities. Tai et al. (2004) demonstrated that there was no significant suppression of nitric oxide (NO) production after induction with lipopolysaccharides (LPS) or cytokine expression in mouse cells (Tai et al., 2004). However, other studies confirmed that these extracts exhibited hydrogen scavenging potential at concentrations as low as 10 µg/mL and therefore did display significant antioxidant potential (Fernandes et al., 2004; Katerere and Eloff, 2005). Another investigation into the antioxidant potential of *S. frutescens* showed that solvents used to prepare *S. frutescens* extracts influenced the ROS scavenging capacity of the extracts (Tobwala et al., 2014). Jiang et al. showed that ethanolic extracts of *S. frutescens* suppressed the induction of ROS production in neurons and microglial cells through inhibition of the interferon gamma (IFN-γ) induced phosphorylated-extracellular signal-regulated kinase (p-ERK)1/2 signalling pathway. This pathway plays a central role in many metabolic processes which in turn suggest that the ethanolic extract of *S. frutescens* may have anti-inflammatory properties through the prevention of tissue damage caused by ROS (Jiang et al., 2014). Similar results were obtained in a study by Lei et al. and although their study confirmed that a *S. frutescens* extract could suppress NO production through IFN-γ induced p-ERK1/2 signalling pathways, neither the sutherlandins (A-D) nor sutherlandiosides (SUA-D) induced a similar effect (Lei et al., 2014). It was suggested that chlorophyll, present in the leave extracts, contribute to the anti-inflammatory effect of a crude ethanolic leaf extract (Lei et al., 2015a). On the other hand, mice, fed a diet containing *S. frutescens*, showed no significant modification of host inflammatory response towards either gram negative or gram positive bacteria (Lei et al., 2016).

One of the few studies demonstrating the effect of *S. frutescens* on gene transcription analyzed the up- and down-regulation of LPS and IFN-γ stimulated genes in a murine macrophage cell line. *S. frutescens* treatment differentially influenced expressed genes (which are involved in inflammatory signalling pathways, such as NF-kB and mitogen-activated protein kinases (MAPK), and resulted in approximately 38 % of the immune stimulated genes being modulated in terms of up- or down-regulation of their expression (Lei et al., 2015b).

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In addition, it has been shown that high concentrations of these extracts (6 mg/mL) increase cellular metabolism resulting in the increased production of reducing equivalents such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). The reducing equivalents donate electrons to the electron transport chain which leads to increased adenosine triphosphate (ATP) production which is utilized for the active reabsorption of nutrients in the kidney. However, the increased flux of ions along the electron transport chain depolarizes the mitochondrial membrane and ROS are produced. This effect thus makes the mitochondria in the proximal convoluted tubule and the distal convoluted tubule susceptive to oxidative stress. The depolarization of the mitochondrial membrane also signals pro-apoptotic factors (Phulukdaree et al., 2010).

The majority of investigations into the potential of *S. frutescens* extracts to be used as anti-inflammatories and anti-oxidants have studied the effects of the whole extracts and only select studies have considered individual compounds that may elicit these effects. It has been shown that the anti-oxidant capacity of L-canavanine is comparable to that of curcumin and that D-pinitol was able to decrease extracellular prostaglandin levels in macrophages by 14 % (Caires et al., 2011). Further immunomodulating effects where observed in the presence of polysaccharides isolated from *S. frutescens* extracts. It was shown that mixtures containing high molecular weight polysaccharides showed greater inhibitory effects compared to low molecular weight polysaccharides when assayed in the complement fixation test (Zhang et al., 2014).

Glucocorticoids play a major role during the inflammatory response and influence pro-inflammatory and anti-inflammatory gene transcription via the GR. In addition to their effects on the inflammatory response, exposure to elevated glucocorticoid levels has also been linked to depression and stress. To our knowledge, there are no previously published studies that specifically investigated the effect of *S. frutescens* extracts on glucocorticoid production in adrenal steroidogenesis and/or the influence of these extracts on GR mediated gene transcription. However, there are several compounds, such as Compound A and ginsenosides, which have been shown to influence the GR mediated effects (de Bosscher et al., 2005; Du et al., 2011; Lee et al., 1997).

Inflammation has long been known to affect the progression of several cancers and although conventional cancer treatments include chemo-and/or radiotherapy or surgery, it has been shown that one in seven malignancies can be linked to chronic inflammation or infection. The

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inflammatory mechanisms that have been linked to the progression of cancers include anti-apoptotic proteins, invasive and metastatic genes, promoters of cell proliferation and the down regulation of some transcription factors. The realization that cancer is a complex disease that may depend on several factors has led to the investigation of multi-targeting therapies, many of which originate from natural sources (Yadav et al., 2010).

Cancer

The anti-proliferative effect of *S. frutescens* was demonstrated by the addition of the extract to several human carcinoma cell lines, including Jurkat, MCF-7, HL-60 and MDA-MB-468 cells (Tai et al., 2004). Other studies confirmed that *S. frutescens* extracts inhibited mammary adenocarcinoma proliferation of MCF-7 cells by approximately 50 % while also inhibiting the growth and changing morphology of the cells (Stander et al., 2007; Vorster et al., 2012). A comparison of the results obtained by the apoptotic studies in MCF-7 cells indicate that the ethanolic extract may contain compounds that are more effective in inducing apoptosis compared to the aqueous extract. In addition, the extract was found to affect the expression of several differentially expressed genes, including those expressing the tumor necrosis factor receptor superfamily (Stander et al., 2007). Morphological disintegration and changes in cellular membranes of Jurkat, Chinese Hamster Ovarian (CHO) and Caski cell lines further demonstrated the apoptotic effects of *S. frutescens* (Chinkwo, 2005), while apoptosis promoting effects were confirmed in lymphocytes (Korb et al., 2010). Esophageal cancer cells treated with *S. frutescens* also displayed typical signs of apoptosis such as cell shrinkage and plasma membrane blebbing (Skerman et al., 2011).

Although earlier investigations showed the anti-cancer effects of *S. frutescens*, studies in recent years have focused on potential signalling pathways that may be affected by the extracts. In addition to nuclear condensation and membrane integrity, Leisching et al. showed that an ethanolic extract of *S. frutescens* affected the phosphorylation state of important molecules in the phosphoinositide 3-kinase (PI3-K) signalling pathway, thereby inducing apoptosis (Leisching et al., 2015). As the PI3-K signalling pathway is linked to the progression of cancer, the latter study suggested that *S. frutescens* may have therapeutic application in various cancers (Sokolowski et al., 2016).

S. frutescens extracts appeared to influence the progression of prostate cancer as the extract was able to repress approximately 50 % of Hedgehog-signalling responsive genes in TRAMPC2 cells (Lu et al., 2015). As Hedgehog signalling has been associated with advanced

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prostate cancer, as well as other cancers and metastases, the repression of genes within this pathway by *S. frutescens* has significant implications in potential cancer treatments. In addition, TRAMP mice fed a diet containing *S. frutescens*, up to 1 % w/w, were shown to have reduced formation of prostate carcinoma. The compounds within the extracts were also assayed for repressive activity toward Hedgehog-signalling genes and it was found that SUD was the more potent contributor to the repressive effect of the extract (Lin et al., 2016; Lu et al., 2015). Further confirmation that the apoptotic activity of *S. frutescens* could be attributed to interference with signalling pathways was obtained when van der Walt et al. showed that an ethanolic extract induced the activation of caspases (CASPs) 8, 9 and 3/7, as well as increased expression of CASP9 and FAS ligand (FASLG) genes, all of which have been associated with apoptosis (van der Walt et al., 2016).

Although the anti-mutagenic effect of *S. frutescens* had previously been demonstrated using the Ames test, a test designed to determine the mutagenic potential of compounds (Reid et al., 2006), this effect was recently confirmed in the presence of tobacco specific carcinogens (Gelderblom et al., 2017). Although it was suggested that the sutherlandins and SUs may be responsible for the anti-mutagenic effect of the extract, these compounds have not, to date, been associated with anti-mutagenic activity. Furthermore, the tobacco specific carcinogens investigated in this study could be inactivated through interfering with P450 enzymes as it has previously been shown that *S. frutescens* can inhibit progesterone (PROG) and pregnenolone (PREG) metabolism by P450 enzymes. Furthermore, recent studies have shown that disrupted endocrine systems play major roles in the development of cancers as well as cancer relapses caused by endocrine resistance that had developed due to chemotherapies (Casco and Soto-Vega, 2016).

Stress and depression

Literature suggests that the use of *S. frutescens* extracts has commonly been linked to stress-relieving effects with anecdotal reports by native tribes having used extracts made from the dried leaves to treat victims of shock, trauma and severe depression (van Wyk, 2011; van Wyk and Albrecht, 2008). Stress-related disorders often relate to a dysfunctioning endocrine system as the production of the human glucocorticoid, cortisol, plays a major role in the response to stress.

The stress response is regulated by the HPA-axis under the control of corticotrophin release hormone (CRH) and adrenocorticotropic hormone release from the brain in reaction to stimuli

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such as stress, hunger or inflammation. Both hunger (Ott et al., 2011) and inflammation (Straub et al., 2011) cause persistent activation of the HPA-axis, whereas stress responses have been shown to vary considerably between individuals (Trestman et al., 1991). Activation of the HPA-axis leads to increased cortisol production and secretion from the adrenal gland, however, glucocorticoids also exert an inhibitory feedback effect on the HPA-axis so that the body can return to a homeostatic state once the stress stimuli has dissipated. In a diseased or chronic stress state, the feedback inhibition by glucocorticoids is no longer effective resulting in elevated circulating glucocorticoid levels (Silverman and Sternberg, 2012). Although the stress related increase in glucocorticoids is initially beneficial, chronically elevated glucocorticoid levels lead to chronic inflammation, heart disease (Adameova et al., 2009), diabetes (Rosmond, 2003), cancers, osteoporosis and an array of psychiatric disorders (Kadmiel and Cidlowski, 2013).

Prevoo et al. suggested that *S. frutescens* could inhibit glucocorticoid production due to the inhibitory effect the extract exhibited towards the catalytic activity of adrenal steroidogenic P450 enzymes, P450 17α-hydroxylase/17,20-lyase (CYP17A1) and P450 21-hydroxylase (CYP21A2) (Prevoo et al., 2004, 2008). The study showed that the extract inhibited steroid substrate binding to CYP21A2 and CYP17A1 in adrenal microsomal preparations, however, the triterpenoid fraction of the extract did not significantly inhibit PREG binding at the concentrations assayed. The triterpenoid fraction used in this study may have been a mixture of SU A-D (Fu et al., 2008) and other SUs that have recently been identified in extracts (Zaki et al., 2016). An *in vivo* study showed that corticosterone (CORT) levels in stressed rats were significantly reduced by the presence of the extracts, together with reduced testosterone and an overall reduced CORT:testosterone ratio (Smith and Myburgh, 2004a). The latter finding was significant with regard to the use of *S. frutescens* extract as treatments in anxiety disorders and cancers as changes in the cortisol:testosterone ratios in humans may affect behavioral changes (Romero-Martínez et al., 2013) as well as the progression of some cancers (Fabre et al., 2016). Smith and van Vuuren, 2014, showed in an *in vivo* study that *S. frutescens* extracts affected steroidogenesis increasing plasma CORT levels in male Wistar rats following a single dose. In addition, the latter study showed that the down regulation of the GR, induced by stress, was eliminated after administration of low dosages (4mg/kg body weight) of *S. frutescens* extract (Smith and van Vuuren, 2014).

Although the reduction of circulating glucocorticoid levels appears to be a significant target for treating stress and inflammatory related disorders, the actions of the glucocorticoids at

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target tissues remain a very important factor in the treatment of these disorders. It is therefore not uncommon for chronic stress to induce disorders such as insulin resistance, obesity and Type 2 diabetes (Black, 2006).

Diabetes

Diabetes is generally associated with insulin resistance through which glucose uptake from circulation is inhibited, however low grade chronic inflammation may also contribute to the development of Type 2 diabetes (Guo et al., 2015). Insulin resistance is a known side effect of glucocorticoid excess and is a common symptom of glucocorticoid excess disorders such as Cushing's syndrome. Although many potential targets have been identified for the mechanism through which glucocorticoids induce insulin resistance, malfunctioning pancreatic beta cells as well as the GR and MR have been investigated. In the context of this study, the potential effect of *S. frutescens* on diabetes mellitus (Type 2) will be discussed as traditional infusions prepared from the leaves of this plant have been used to treat this clinical condition.

In vivo studies have shown that in streptozotocin induced diabetic rats, blood glucose concentrations were decreased after administration of *S. frutescens* (800 mg/ kg bodyweight). In addition, these rats maintained lowered blood glucose concentration longer than a conventional hypoglycemic agent (chlorpropamide) (Ojewole, 2004). Chadwick et al. showed that rats fed a high fat diet that induced insulin resistance displayed improved glucose clearance after the administration of *S. frutescens* (Chadwick et al., 2007). Furthermore, it was shown that *S. frutescens* administration to insulin resistant mice increased glucose uptake in the muscle significantly, and although increased, the glucose uptake in adipose tissue and liver was not significantly different to the control group. These studies also showed that the extract reduced free fatty acids and triglycerides in circulation when compared to the control group, together with reducing circulating free fatty acids when compared to the non-insulin resistant control group (Mackenzie et al., 2009; MacKenzie et al., 2012).

The effect of *S. frutescens* on hepatocytes was investigated by assessing parameters descriptive of insulin resistance such as deoxy-glucose uptake, glucose secretion into the culture medium and intracellular lipid accumulation. The treatment of the cells with *S. frutescens* affected the transcriptional activities of 27 potential gene targets for the treatment of insulin resistance. The influence of the extract on these genes suggests various likely mechanisms through which *S. frutescens* may exert its anti-diabetic effects (Williams et al., 2013). Several compounds within the extract may contribute to the anti-diabetic effects observed, although earlier studies

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investigating *S. frutescens* as a potential anti-diabetic treatment suggested that pinitol and L-canavanine within the plant extracts may be responsible for the anti-diabetic effects (Sia, 2004).

Immunodeficiency disorders

The traditional use of *S. frutescens* as an immunostimulating tonic for immunocompromised individuals, such as human immunodeficiency virus (HIV) patients, has been recognized by the South African Ministry of Health (Mills et al., 2005b), despite little conclusive scientific data. Although initial investigations indicated that the use of *S. frutescens* may be beneficial, recent data have suggested that extracts may have detrimental effects.

Studies first showed that a methanolic *S. frutescens* extract stimulated recombinant HIV-1 reverse transcriptase (RT) activity (Bessong et al., 2005), while Harnett et al. demonstrated that *S. frutescens* extracts inhibited the HIV-1 RT activity and suggested that the tannins present in the extract may contribute to the inhibitory effect (Harnett et al., 2005). In addition to these conflicting results, it was found that the use of extract may affect anti-retroviral drug metabolism as *S. frutescens* was found to inhibit P450 3A4 (CYP3A4), a hepatic cytochrome P450 enzyme involved in drug metabolism (Mills et al., 2005a).

Further investigations led to the realization that the extracts may induce apoptosis in cluster of differentiation (CD) 4^+ cells and T lymphocytes, as well as elevating the concentration of cells expressing CD69, suggesting that the observed apoptosis may be due to activation-induced lymphocyte cell death (Korb et al., 2010). As immunocompromised individuals have low $CD4^+$ cell counts, further reduction of these cells would aggravate their condition. However, Wilson et al. demonstrated that HIV viral load and $CD4^+$ cells and T lymphocyte counts were not significantly different in HIV seropositive individuals receiving *S. frutescens* when compared to the control group (HIV positive, but not receiving *S. frutescens*). The total burden of infection, quantified as the number of days of infection-related occurrences, was significantly greater in the group receiving *S. frutescens* with two subjects contracting tuberculosis while receiving isoniazid preventative therapy (Wilson et al., 2015). This effect may be due to the anti-oxidant and anti-inflammatory effects of the extract which, in a healthy individual, would be beneficial. However, in an immunocompromised individual the anti-inflammatory effect may reduce the host's immune system further and in doing so might advance latent infections such as caused by *Mycobacterium tuberculosis* (Folk et al., 2016). However, a recent study has shown that *S. frutescens* and alpha-linolenic acid, isolated from the extracts, inhibited the metabolic action of shikimate kinase, a key enzyme in the metabolic pathway of *M.*

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tuberculosis. A dichloromethane extract showed the greatest inhibitory effect (IC_{50}= 0.1 µg/mL) when comparing extraction methods, whereas alpha-linolenic acid showed a inhibitory effect (IC_{50} = 3.7 µg/mL) comparable to that of the ethanolic or aqueous *S. frutescens* extracts (IC_{50} = 1.7 and 5.1 µg/mL) (Masoko et al., 2016). In addition, under immunocompromised conditions, *S. frutescens* appears to aggravate the inflammatory response by further enhancing Jun amino-terminal kinases (JNK)-activated pro-inflammatory pathways. Africa and Smith showed that *S. frutescens* results in increased inflammatory leukocyte movement across the blood brain barrier in co-cultured HL2/3 and blood brain barrier cells (Africa and Smith, 2015).

Although extracts have been shown to have significant effects in the aforementioned diseases, clinical conditions and *in vitro* studies, investigations into specific compounds identified within *S. frutescens* extracts and the association of these compounds with particular effects are limited. As mentioned previously, neither the SUs nor sutherlandins displayed potential as an antioxidant, however the repression of prostate cancer specific genes by SUD suggests a potential anticancer application. Furthermore, D-pinitol has been associated with anti-diabetic effects and with decreased extracellular prostaglandin levels in macrophages. In addition to these compounds, a number of other compounds have been identified in *S. frutescens* extracts and are discussed next.

Compounds identified within *S. frutescens* extracts

The medicinal effects of *S. frutescens* have been attributed to a variety of compounds that are present in extracts. It has been demonstrated that the pharmacological composition of the extracts may be influenced by the geographic location of the plant, harvesting and extraction methods and therefore extracts and infusions made from these plants may have varying medicinal effects. As specific compounds within extracts have been associated with specific medicinal effects, the analyses of extracts and the quantification of specific compounds in different populations, affected by growth conditions and by harvesting methods, is crucial to studies investigating bioactivities of *S. frutescens*.

Amino acids

Amino acids are abundantly present in extracts and quantitation analyses showed that all 20 naturally occurring amino acids are found in *S. frutescens* extracts. In addition to these amino acids, GABA, L-canavanine, L-arginine, L-asparagine and D-pinitol have been identified to be major constituents in extracts. Many of the therapeutic applications of *S. frutescens* have been associated with the presence of these compounds. Alanine, proline and L-asparagine were

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shown to represent approximately 60 % of the total amino acid quantified in the extracts (Mncwangi and Viljoen, 2012). While the non-protein amino acid L-canavanine is ±0.08 mg/g in leaf extracts, D-pinitol ± 18 mg/g was present at the highest concentration in leaf extracts, followed by GABA ± 3.5 mg/g. GABA was present at ± 1.7 mg/g in seed extract, and D-pinitol was present in the lowest concentrations ± 0.25 mg/g) (Shaik et al., 2010). Although surprising, the GABA levels were lower in the seed extracts than in the leaf extracts which may be explained by the tendency of GABA levels to remain low in the seeds of legumes and to be rapidly increased after germination (Kuo et al., 2004).

L-canavanine, associated with anti-cancer effects of *S. frutescens*, displays toxic effects due to its ability to interfere with the interaction between arginine and arginyl tRNA synthetase, since L-canavanine and arginine are structurally similar (Fig 2.1A). However, the inhibition of arginine metabolizing enzymes by L-canavanine may only occur when arginine concentrations are low and the low concentrations at which L-canavanine in *S. frutescens* has been reported suggests that it would not contribute significantly to toxicity of *S. frutescens* (Shaik et al., 2010). In addition, the inhibition of arginine metabolism may affect nitrogen uptake and in so doing add to the anti-oxidant potential of the extracts by reducing the production of NO (Caires et al., 2011). It has also been shown that arginine substitution by L-canavanine might be more effective under inflammatory conditions and may also have an influence on autoimmunity (Akaogi et al., 2006).

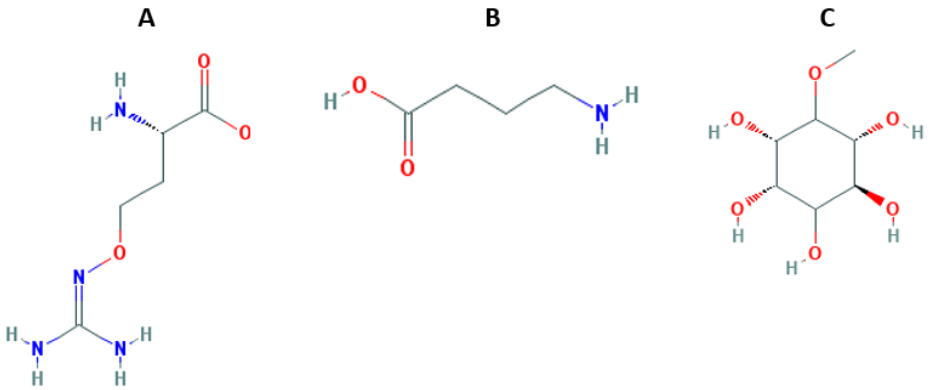

Fig. 2.1: Chemical structures of A) L-canavanine[a], B) GABA[b] and C) D-pinitol[c].

[a] National Center for Biotechnology Information. PubChem Compound Database; CID=439202, https://pubchem.ncbi.nlm.nih.gov/compound/439202

[b] National Center for Biotechnology Information. PubChem Compound Database; CID=119, https://pubchem.ncbi.nlm.nih.gov/compound/119

[c] National Center for Biotechnology Information. PubChem Compound Database; CID=164619, https://pubchem.ncbi.nlm.nih.gov/compound/164619

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GABA (Fig 2.1B), an inhibitory neurotransmitter, has been associated with the anti-anxiety and sedative effects of *S. frutescens* and studies have shown that mood disorders can often be associated with reduced GABA levels (Abdou et al., 2006). Furthermore, GABA has been shown to increase immunoglobulin (Ig) A levels, thereby enhancing the immune system during stress. Outside of the central nervous system (CNS) GABA has been shown to be involved in the inhibition of tumor cell migration (Ortega, 2003), as well as influencing steroid production through affecting the HPA-axis (Cullinan et al., 2008; Metzeler et al., 2004).

D-pinitol, shown in Fig. 2.1C, has been associated with the anti-diabetic effects of *S. frutescens* and reports have shown that D-pinitol is able to induce insulin-like effects in streptozotosin-induced diabetic rats as well as displaying anti-hyperglycemic effects (Bates et al., 2000). In addition to reports showing the favourable effect of D-pinitol on glucose uptake, this compound has been shown to display anti-inflammatory (Singh et al., 2001) and anti-hyperlipidemic (Geethan and Prince, 2008) effects. The latter study showed that D-pinitol decreased blood glucose levels, as well as free fatty acids, triglycerides, total cholesterol and phospholipids. Although D-pinitol has mostly been associated with anti-diabetic effects, studies have shown that the presence of this compound in aqueous *S. frutescens* extracts may decrease the absorption of atazanavir, an anti-retroviral drug, in Caco-2 cells (Müller et al., 2012).

Flavonoids and triterpenoids

The polyphenol fraction of plant extracts is known to contain a range of compounds, most of which are yet to be identified. These compounds also elicit a diverse range of medicinal effects, however in the scope of this thesis, only compounds that have been identified within *S. frutescens* extracts will be discussed.

Flavonoids, which have been classified into flavones, isoflavones, flavanols, flavan-3-ols and anthocyanidins, are naturally abundant in plants as bioactive secondary metabolites. The effect of flavonoids has been a popular topic of investigation in recent years and it has been shown that these compounds show anti-inflammatory effects by suppressing NF-κB production after the onset of LPS-induced inflammation (Funakoshi-Tago et al., 2016). In addition, flavonoids have been identified as a potential therapy to treat intestinal inflammation associated with obesity (Gil-Cardoso et al., 2016). The anti-oxidant activity has been attributed to the inhibitory effect of the flavonoids on LPS-signalling pathways (Chanput et al., 2016). The recent implication of flavonoids in cardio-protective functions have shown that specific structural characteristics allow for improved cardio-protective effects directly after myocardial

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ischemia/reperfusions in rats (Testai et al., 2013). In addition, Zhang et al. showed that flavonoids possess hepato-protective properties (Zhang et al., 2014). Although flavonoid compounds within *S. frutescens* extract have been shown to be present in relatively low concentrations (Shaik et al., 2011), the sutherlandins A-D (Fig 2.2) have also been identified within the *S. frutescens* extracts (Avula et al., 2010).

Very little data is available regarding the effects of the sutherlandins specifically in anti-inflammatory, cardio-protective or hepato-protective processes. Although Tobwala et al. showed that a decrease in the flavonoid content of the extract resulted in a decrease in ROS scavenging potential of the extract (Tobwala et al., 2014), a subsequent investigation showed that sutherlandins A-D and SU A-D contributed very little to the anti-inflammatory effects observed when murine macrophages were exposed to ethanolic *S. frutescens* extracts (Lei et al., 2015a).

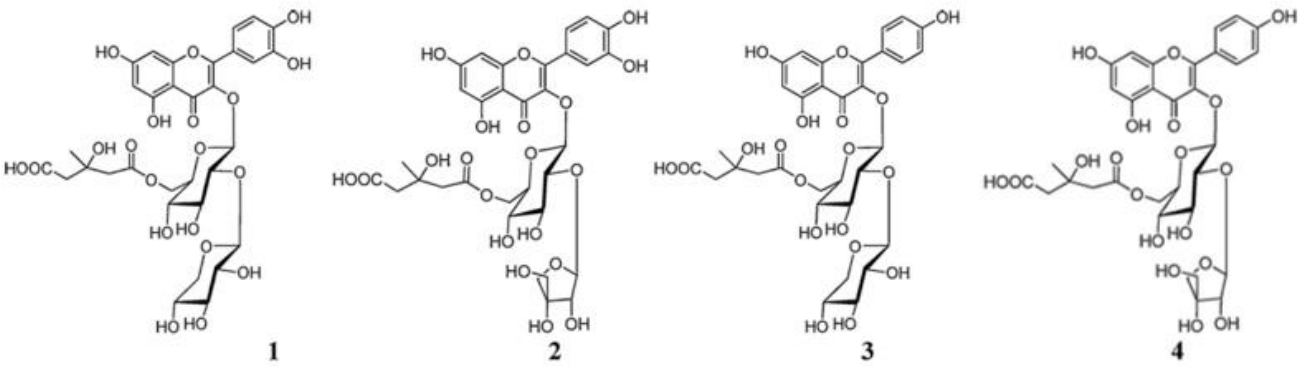

Fig. 2.2: Chemical structures of sutherlandins 1) A, 2) B, 3) C and 4) D identified in *S. frutescens* extracts. Reproduced from Avula et al., 2010.

S. frutescens extract have also been shown to contain triterpenoid compounds. These compounds are present in varying concentrations in plant extracts and have been linked to a number of medicinal properties. SUB has been identified as the major triterpenoid in *S. frutescens* extracts, followed by SU A, C and D (Fig 2.3) (Albrecht et al., 2012; Avula et al., 2010; Brownstein et al., 2015; Fu et al., 2010, 2008; Olivier et al., 2009). Studies have shown that triterpenoids are able to inhibit nociceptive responses (Wu et al., 2011) and elicit anti-inflammatory, cardio-protective (Bishayee et al., 2013; Han and Bakovic, 2015) and anti-cancer effects (Bai et al., 2016; Yadav et al., 2010). It has recently been shown that ursolic acid, a pentacyclic triterpenoid carboxylic acid, displays both anti- and pro-inflammatory effects (Babalola and Shode, 2013; Ikeda et al., 2008).

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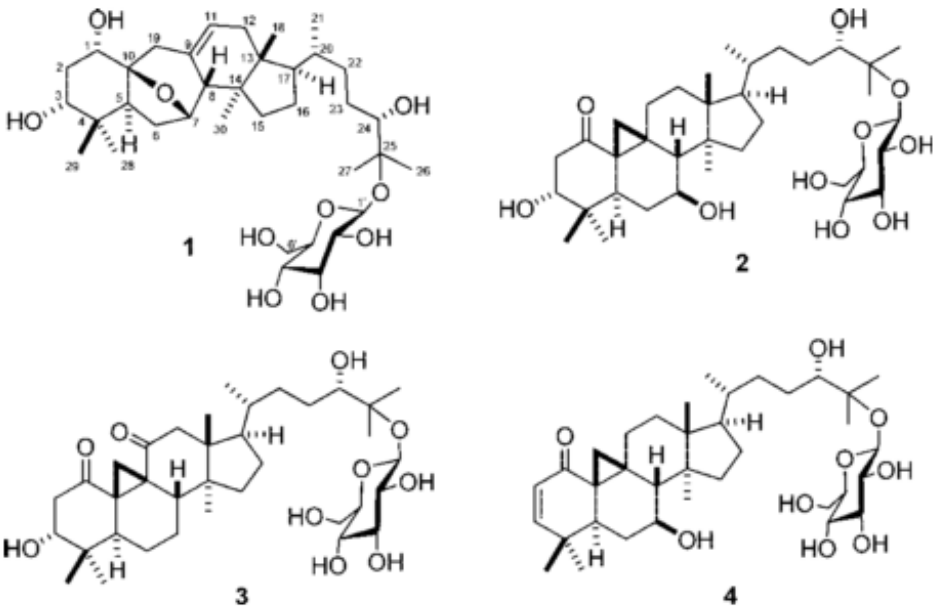

Fig. 2.3: Chemical structures of SU A-D (1-4) isolated from *S. frutescens* extracts. Reproduced from Fu et al., 2008.

Although limited data regarding the effect of SUs on anti-inflammatory, cardio-protective or anti-cancer properties generally associated with triterpenoids is available, recent studies have shown that anti-cancer effect of *S. frutescens* could be attributed, in part, to the presence of SUD as this SU could suppress the Gli/hedgehog signalling pathway in prostate cancer cells (Lin et al., 2016). Furthermore, studies have shown that the triterpenoid glycoside fraction obtained from a *S. frutescens* extract affected atazanavir metabolism in human liver microsomes, indicating a possible herb-drug interaction (Müller et al., 2012). The fact that the structures of the SUs are similar to that of steroid hormones and steroid hormone metabolites, may suggest that these compounds can elicit biological effects through interactions with steroidogenic enzymes and steroid receptors. This interaction may partly explain anti-inflammatory and cardio-protective properties, however no data has been published to support this.

Other bioactive components.

As plant extracts naturally contain many structurally and functionally diverse compounds, all of these compounds have not yet been investigated for biological activities. Compounds, other than that discussed in previous sections, which have been identified in *S. frutescens* extracts and shown to possess biological activities, are described here.

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Polysaccharide fractions of *S. frutescens* extracts were shown to possess immunomodulating properties when assayed in the complement fixating activities. High molecular weight polysaccharide fractions, fractions containing higher concentrations of xylose and galactose-rich fractions, displayed higher bioactivity compared to the other fractions assayed. Treating the polysaccharide fractions with pectinase also resulted in higher biological activities when compared to untreated fractions (Zhang et al., 2014).

Recently, alpha-linolenic acid was identified and isolated from *S. frutescens* extracts. The identification of this compound in the extract has prompted investigations into the anti-tuberculosis effects of *S. frutescens* as this extract was shown to inhibit recombinant shikimate kinase which is considered to be an effective drug target for the development of anti-tuberculosis drugs (Masoko et al., 2016).

Cardenolides, cardiac glycosides (Fig 2.4) that are used as protection against herbivores in plants, have recently been identified in leaf and seed extracts of *S. frutescens.* The presence of these compounds suggests that the extracts may have cardio-protective properties as these compounds have been used extensively to treat congestive heart failure (Braga et al., 1996; Shaik et al., 2011). The use of cardenolides to treat heart failure may be rooted in their ability to affect ion transport mediated by sodium-potassium adenosine triphosphatase (Na^+,K^+-ATPase) (Florkiewicz et al., 1998). Furthermore, cardenolides have recently been shown to play a significant role in the treatment of non-small cell lung cancer as these compounds induce lysosomal membrane permeability (Mijatovic et al., 2006).

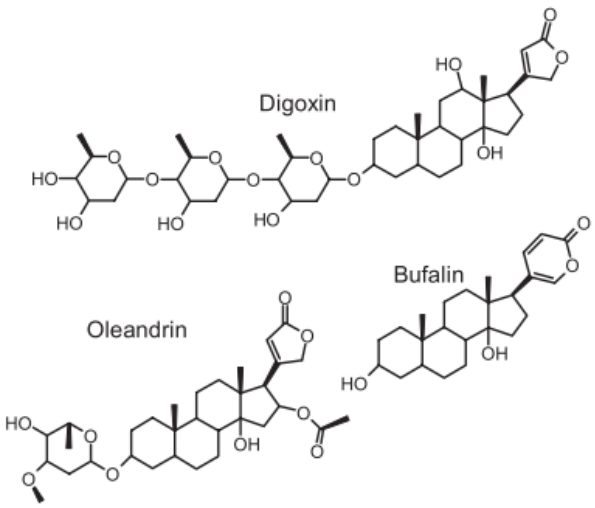

Fig. 2.4: Typical cardenolide structures. Reproduced from Schoner and Scheiner-Bobis, 2007.

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Bioavailability of compounds

Many bioactive compounds have been shown to elicit various medicinal effects, however, in most cases these studies have been conducted *in vitro* and little *in vivo* data is available. In addition to this, it is not uncommon for an effect observed *in vitro* to be abolished *in vivo*. The difference observed *in vivo* and *in vitro* can be attributed to the bioavailability of the natural compound in the target tissue. As most herbal remedies are taken orally, these compounds would be subjected to digestion, metabolism by gut flora and hepatic enzymes and potential transport within circulation. It is therefore not uncommon that compounds do not reach their intended target in the active form assayed, if these compounds reach these targets at all. The bioavailability, absorption and degradation of compounds within plant extracts after oral ingestion will thus affect their overall biological activities.

Lipinski's rule of five has been used as a guideline to identify potential new drugs that would be bioavailable after oral intake. This rule describes four physicochemical characteristics describing limitations for molecular weight ($\leq$ 500 Da), lipophilicity (log P $\leq$ 5) and the number of hydrogen donors and acceptors (H-bond donors $\leq$ 5 and H-bond acceptors $\leq$ 10) (Lipinski, 2004). These characteristics are used to predict the aqueous solubility and intestinal permeability of compounds and have been linked to ± 90 % of oral medications that have reached phase II clinical trials. The use of this rule has however impacted drug discovery and development as many potential drugs that may not adhere to these rules, have been overlooked even though Lipinski himself cautioned against applying the rule unilaterally.

Compounds with relatively simple chemical structures found within *S. frutescens* extracts are most likely to be readily available and to be absorbed from the intestines. GABA, D-pinitol and L-canavanine are amino acids and it is likely that these compounds would therefore undergo the same absorption and metabolism as natural amino acids. However, the more complex molecules within the *S. frutescens* extracts, such as flavonoids and triterpenoids, may be less likely to be absorbed in the structure that they have been assayed in *in vitro* assays (Thwaites et al., 2000).

A recent study has shown that a single dose of 300 mg of *S. frutescens* could result in a gastro-intestinal (GI) concentration of extract remarkably greater than the minimum concentration required for the inhibition of hepatic P450 enzymes, while the greatest inhibitory effect was observed for metabolism by CYP3A4 and 3A5 (Fasinu et al., 2013). The interaction of triterpenoids with P450 enzymes is not surprising as these compounds are synthesized by plant

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P450 enzymes in higher plants (Yamazaki, 2014). Other studies indicated that protopanaxatriol (PPT)-type ginsenosides are metabolized by P450 enzymes, specifically CYP3A4, in rat and human liver microsomes (Etheridge et al., 2007). Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-dependent metabolism by P450 enzymes showed that the major site of oxygenation of the triterpenoids and aglycone is on the C20 aliphatic branch side-chain. Furthermore, C20-24 epoxides were produced by oxygenation of the C24-25 double bond. These oxygenation reactions appear to play significant roles in the elimination of ginsenosides, however the specific P450 enzyme isoforms responsible for these reactions are unclear as these experiments were performed in microsomal fractions (Hao et al., 2010).

It has been shown that plasma triterpenoid levels remain low after oral administration due to extensive metabolism in the GI tract, poor membrane permeability and the low solubility of the triterpenoid aglycone (Tawab et al., 2003). After ingestion triterpenoids, such as ginsenosides, are metabolized in the gut by intestinal bacteria, often resulting in the deglycosylation of the molecule via subsequent sugar moiety cleavage reactions (Kim et al., 2013; Qi et al., 2011). The digestion and absorption of triterpenoids in Wistar rats was investigated by feeding rats glycosidic extracts of *Saraitia grosvenori* and it was shown that mogroside V, a triterpenoid isolated from *S. grosvenori,* and its metabolites were found in the small intestine and the blood samples collected. Mogroside V had been degraded to tetra and triglucose mogroside conjugates and no free triterpenoids were detected. Approximately 60% of the triterpenoids administered were detected in the fecal matter collected 24 hours after administration (Murata et al., 2010). This confirms the low absorption of triterpenoids from the small intestine that has previously been shown. Previous studies have also shown that glycoside compounds can be detected as glucuronic and sulfuric acid conjugates *in vivo* after oral administration to rats (Moon et al., 2000; Piskula, 2000).

Studies have shown that the association of monosaccharide and disaccharide moieties to triterpenoid compounds, specifically at C3 and C28, influence their pharmacokinetic parameters (Tang et al., 2015). Although the pre-systematic metabolism of ginsenosides may result in relatively low plasma levels, it has been shown than these triterpenoids are present in hepatic tissues at much higher concentrations than plasma and other tissues (Gu et al., 2009). In addition, the analyses of ileal fluids after the ingestion of raspberries showed the presence of β-courmaroyl glucarate derivatives and thus the ability of the compounds to be delivered to target tissues and elicits drug-like effects (McDougall et al., 2016). It has been also been shown that the addition of small molecule fractions of extracts to steroidal saponins increases the

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bioavailability of these compounds which may explain the relatively high bioavailability of whole plant extracts and limited bioavailability of triterpenoid compounds (Tang et al., 2015). Further technologies have also been developed to increase the bioavailability of compounds. These include the application of nano-sized drug carrier particles including liposomes and micelles, while cyclodextration creates a pseudo-amphiphilic effect to increase bioavailability (Aqil et al., 2013; Krishnaiah, 2010).

Potential drug-herb interactions

The interactions between drugs and herbs are of great significance, especially since herbal remedies are being used more frequently in conjunction with pharmaceuticals. The majority of traditional medicines have not been screened for drug-herb interactions and due to the variety of compounds present in traditional medicine, it is plausible that these medicines may interfere if taken in conjunction with drugs and elicit unfavourable effects. Although the majority of drug-herb interactions have been associated with negative effects, these interactions could potentially increase the effects of the drug or decrease adverse side effects associated with the use of the drug. A well-documented example of a synergistic drug-herb interaction is the use of garlic and captopril which resulted in decreased blood pressure and a greater inhibitory effect on angiotensin-converting enzyme (Asdaq and Inamdar, 2010).

The most common drug-herb interactions are associated with pharmacokinetic effects often resulting in changes in pharmacokinetic parameters, plasma concentrations or elimination half-life of pharmaceuticals (Shi and Klotz, 2012; Valli and Giardina, 2002). These effects are often elicited either through the inhibition or stimulation of hepatic P450 enzymes or potential drug carriers in circulation. The most common method of pharmacokinetic interference is the competitive inhibition of the metabolizing enzyme through the binding of a herbal medicine or compound to the active site of the enzyme (Pelkonen et al., 2008). Approximately 70 % of currently used drugs are metabolized by only ten P450 isoforms of which CYP3A4 is the most abundant and is responsible for the metabolism of the majority of drugs and xenobiotic currently in use (Pelkonen et al., 2008). CYP3A7 and CYP3A5 are also present in the livers of a small percentage of the Caucasian population and together with CYP2D6, which is much less abundant than CYP3A4, metabolizes numerous drugs. In addition to CYP3A4 and CYP2D6, the oxidative metabolism of drugs is mostly mediated by CYP2B6, CYP2C9 and CYP2C19 (Guengerich, 2008). Most drugs that are metabolized by only one P450 isoform are more susceptible to harmful herb-drug interactions than drugs that are metabolized by numerous P450 isoforms.

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In the majority of cases, the inhibition of hepatic P450 enzymes is the most frequent cause of drug-drug interactions. The P450 enzymes may inhibit the metabolism of pro-drugs to their active form and may, as reported in many cases, subsequently lead to toxicity through the inability of the body to clear the drugs effectively. Although the majority of the interactions of drugs with P450 enzymes may be due to competitive and reversible inhibition, mechanism-based inhibition has also been observed.

The stimulation of P450 enzymes is usually associated with the activation of gene transcription, increased messenger RNA (mRNA) biosynthesis or protein degradation. The mechanisms of induction are slow and generally constitute ligand-dependent activation of transcriptional activities via nuclear receptors, most notably pregnane X, androstane and hydrocarbon receptors (Lin, 2006). CYP3A4, CYP2D6 and CYP1A2 have been shown to be susceptible to herb-mediated induction and may thus result in inefficacy of drugs (Shi and Klotz, 2012).

The effects of drug-herb interactions are not limited to the inhibition or induction of P450 enzymes with potential drug transporters, such as P-glycoprotein located in the gastrointestinal tract, liver and blood brain barrier, being a popular aspect of investigation. The secretion of absorbed drugs has been associated with the actions of P-glycoprotein and it has been shown that this transporter secretes the drugs into the intestinal lumen. Interference with the normal action of this transporter could thus result in elevated or decreased drug levels, depending on the interaction of the herb or drug with the transporter. The fact that many bioactive compounds or drugs act as substrates for P-glycoprotein and CYP3A4 has shown that in this transport-metabolism system transporter proteins may act as a barrier in the efficient uptake of bioactive compounds taken orally (Pal and Mitra, 2006).

In vitro assays utilizing *S. frutescens* extracts showed that the extracts inhibited ATP-binding cassette transporters, including P-glycoprotein, and organic anion transporting polypeptides in cells over expressing these transporters. Furthermore, the extract was found to inhibit CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and CYP3A5, while inhibition of the latter two was the most sensitive to the extracts. Mechanism based inhibition of CYP3A4 was observed in the presence of the extract and the metabolism of midazolam, a bioactive drug administered orally, was also decreased in the presence of the extract, resulting in 40 % less drug clearance (Fasinu et al., 2013). Awortwe et al. however showed that CYP1A2 was more susceptible to inhibition by the extract than CYP3A4 (Awortwe et al., 2014). In addition, SUB has also been shown to interfere with hepatic P450 enzyme action by inhibiting CYP3A4

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without affecting CYP2D6 (Madgula et al., 2008). No investigations into the effect of SUs on drug transporters have been published to date.

In addition to inhibiting hepatic P450 enzymes and drug transporters, *S. frutescens* extract have been shown to affect the metabolism of anti-retroviral drugs. The administration of *S. frutescens* to neverapine treated rats did not result in significantly increased neverapine in plasma, although pharmacokinetic parameters for neverapine were decreased indicating a stimulatory effect of the extract on CYP3A2 (rodents do not express CYP3A4). The induction of CYP3A2 was further indicated by increased intestinal CYP3A2 mRNA levels (Minocha et al., 2011). Taken together with previous studies, this finding suggests that *S. frutescens* may inhibit and/or induce the hepatic drug metabolizing P450 enzymes and would likely be responsible for considerable drug-herb interactions.

Safety and efficacy

In spite of the drug-herb interactions described above, the safety of *S. frutescens* extracts is still of major concern. *In vitro* studies have shown that the extracts elicit apoptotic and cytotoxic effects, however, it should be noted that these studies utilized up to 7.5 mg/mL in cell models (Chinkwo, 2005; Ngcobo et al., 2012; Stander et al., 2007; Vorster et al., 2012). This concentration is relatively high, considering the marketed dosage of *S. frutescens* tablets is 800 mg per day. The latter dosage was utilized in a double-blinded study investigating the safety and efficacy of *S. frutescens* in healthy adults. The use of *S. frutescens* has not been associated with severe side effects, although symptoms such as “cotton” mouth, dizziness and slight diuresis have been reported. In healthy adults, no significant changes were observed for vital signs, blood or biomarker indices when compared to the control group, with elevated appetites being the most significant difference between the treatment and placebo group (Johnson et al., 2007). However, when *S. frutescens* was administered to HIV seropositive adults, the HIV viral load and $CD4^+$ T-lymphocytes were similar in control and treatment groups. In addition, the total burden of infection was significantly greater in the group receiving *S. frutescens* with two subjects contracting tuberculosis while receiving isoniazid preventative therapy (Wilson et al., 2015). This effect may be due to the inhibitory effect of the extract on hepatic P450 enzymes as described above. Although *S. frutescens* has been assumed to be safe for use and data show that the use of the extracts in healthy adults has not been linked to any severe side effects, data regarding effects of the extract in a diseased state is limited.

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Conclusion

The greater majority of the South African population (± 75%) still relies on traditional medicine as either part of or their entire primary healthcare system. Remedies such as *S. frutescens* decoctions are widely used with little or no knowledge about the impact of the constituents of the plant on the disease state. The scientific validation of the use of *S. frutescens* extracts as a therapeutic aid in the supplementary treatment of hypertension, diabetes and stress may stimulate the use of this herbal remedy by the general public. Although many pharmaceuticals are readily available to treat these ailments, pharmaceuticals often elicit non-specific actions which lead to adverse side effects. Since no significant side effects have been associated with the use of *S. frutescens* (Johnson et al., 2007), other than potential interference of the metabolism of conventional drugs, this herbal remedy may provide safe, affordable treatment for the aforementioned ailments as well as in HIV seronegative adults (Wilson et al., 2015).

South Africa is one of the three richest floristic areas in southern Africa and in spite of increasing commercial and scientific interest in traditional herbal medicines, no commercially marketable drug has materialized from the scientific investigations in South Africa. Several indigenous species have been exploited internationally, such as *Streptomyces natalensis, Pelargonium sidoides*, and compounds isolated from these species have been included in various foreign patents (Drewes, 2012). Although there seems to be a vast increase in the scientific validation of the use of herbal medicines, the commercialization and consequential economic enrichment appears to be lacking. It is necessary to promote the sustainable development of the natural plant industry in developing countries such as South Africa. The successful integration between science and traditional medicines has the potential for great economic gain, as well as scientific gain, through the validation of the claims associated with these medicines.

In traditional medicinal practices it is not uncommon to find that traditional healers use a single plant to treat a vast range of different ailments that may seem unrelated, but it is more than likely that there is a common target for all these ailments that may be influenced by one or more of the compounds present in these extracts. This hypothesis may be substantiated by the collection of *in vitro* studies discussed in this chapter, displaying strong association between the targets for anti-inflammatory, anti-oxidant and anti-cancer effects. In addition to these effects, the identification of cardenolides in a *S. frutescens* extract, together with the strong association between cardenolides and anti-inflammatory effects, suggest that the extract and compounds within the extract could be employed in cardio-protective treatments.

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The effects of *S. frutescens* has mostly been demonstrated in studies conducted in simple cell models that target simplified systems. However, disease states are more complex than the models utilized to study the effects of the extracts. Although data from *in vivo* models are limited, the *in vitro* studies that have been conducted show that whole *S. frutescens* extracts, as well as compounds within the extract, exert significant effects at target tissues. Although the mechanism by which degradation, absorption and metabolism of these compounds occur is still largely unknown, the interaction with hepatic P450 enzymes indicate potential metabolic routes impacting on the bioavailability of the compounds within the extracts. Since the extracts inhibited several hepatic P450 enzymes, we hypothesized that *S. frutescens* would also interact with adrenal P450 enzymes. The potential mechanisms of these interactions, the adrenal P450 enzymes and their roles in steroid metabolite production are discussed in the following chapter.

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Chapter 3: Corticosteroid biosynthesis, transport and peripheral effects.

Introduction

The ability of an organism to maintain its internal environment by adjusting physiological processes is essential for survival − a regulatory process referred to as homeostasis. The daily activities of all organisms expose them to perturbations in their internal environment due to food intake, physical exercise, psychological stress, etc. These activities may cause a change in the homeostatic state of the organism and the survival of the organism is highly dependent on its ability to ward off these perturbations and return to a homeostatic state. The corticosteroids, glucocorticoids and mineralocorticoids, play a major role in the maintenance of homeostasis as these hormones are involved in metabolism, inflammatory responses and many cardiovascular functions. The regulation of the biosynthesis, secretion and delivery of corticosteroids to target tissues is thus critical for survival, with the dysfunction of their regulatory mechanisms having detrimental effects (Messaoudi et al., 2012; Spies et al., 2011).

Corticosteroids are biosynthesized in the adrenal gland by adrenal steroidogenic enzymes that include P450 enzymes and hydroxysteroid dehydrogenases (HSDs) (Miller and Auchus, 2011). The major glucocorticoid in humans is cortisol, CORT in rats, and upon receiving a stimulus, the HPA-axis is activated and cortisol biosynthesis and secretion from the adrenal gland increases. Under normal physiological conditions, the elevated cortisol levels will initiate negative feedback inhibition of the HPA-axis and cortisol levels will be decreased to that of the homeostatic state. However, in chronically stressed individuals, the feedback inhibition is impaired and cortisol levels remain elevated (Harris et al., 2013).

The major mineralocorticoid in humans is aldosterone (ALDO) and although the glucocorticoids can also elicit mineralocorticoid effects through interaction with the MR, these effects are mitigated by several protective mechanisms at target tissues. The mineralocorticoids are less versatile than the glucocorticoids, but are responsible for maintaining essential cardiovascular functions (McCurley and Jaffe, 2012; Messaoudi et al., 2012).

In this chapter, adrenal corticosteroid biosynthesis, corticosteroid transport and the interactions of corticosteroids at receptor level are the main points of discussion.

Regulation of corticosteroid biosynthesis

The biosynthesis and secretion of corticosteroids is regulated by many physiological factors; although two main processes are involved in their regulation. Firstly, the circadian rhythm is responsible for the release of the corticosteroids in a daily, rhythmic pattern under normal

physiological conditions. Secondly, the HPA axis is involved in the regulation of the homeostatic state through controlling fluctuations in corticosteroid concentrations due to daily activities, which may include physical exertion, food intake, or stressful stimuli. These corticosteroids ensure the regulation of glucose, protein and lipid metabolism, water and electrolyte balance, pro- and anti-inflammatory responses and the regulation of cardiovascular functions.

Circadian rhythm

The physiology of most organisms differs remarkably between day and night. The circadian clock, an intrinsic biological clock, is both self-sustainable and entrained by external stimuli such as daylight and is largely responsible for the daily, rhythmic corticosteroid release. Three basic systems are involved in maintaining the circadian rhythm: 1) environmental signals such as daylight and food intake (input), 2) the suprachiasmatic nucleus (SCN) that is an intrinsic rhythm generator responsible for the expression of "CLOCK" genes and 3) output rhythms which result in the secretion of corticosteroids (Chung et al., 2011). The regulation of the circadian rhythm is depicted in Fig 3.1.

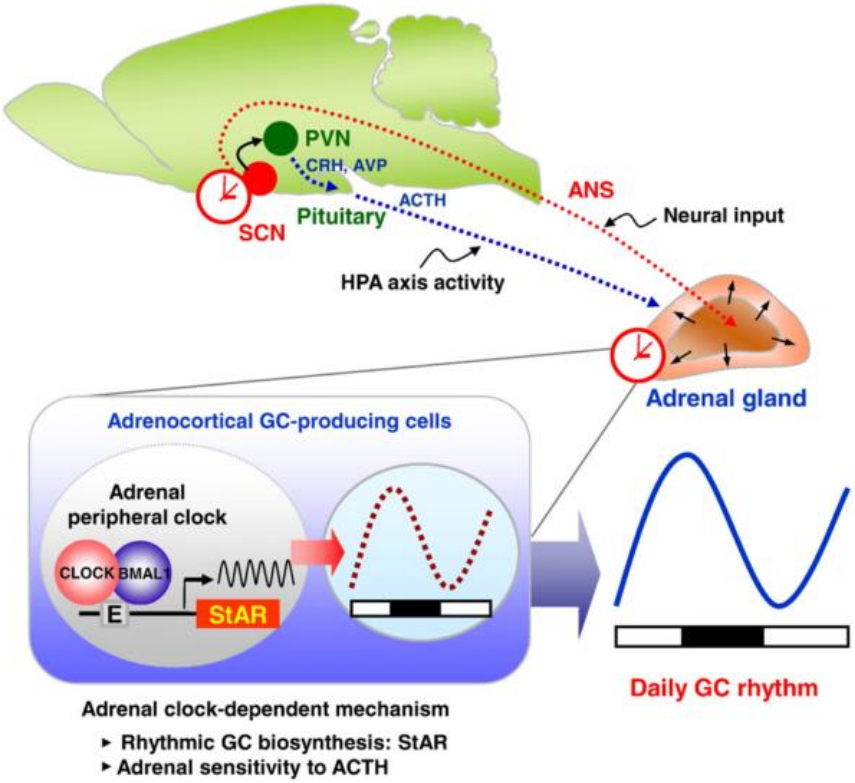

Fig. 3.1: Schematic representation of the regulation of the inputs influencing daily fluctuations in cortisol secretion as part of the circadian rhythm. Reproduced from Chung et al., 2011.

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Circulatory corticosteroid levels are increased during the active periods of the circadian rhythm with the highest corticosteroid concentrations detected at the start of the activity period. The rhythmic secretion of cortisol from the adrenal gland is controlled by the HPA-axis, which in turn is activated in response to stimulation from the SCN (the central clock). The rhythmic production and secretion of arginine vasopressin (AVP) is also under regulation of the SCN. Alternatively, a more local circadian regulatory function involves the stimulation of the adrenal gland by the splanchnic nerve which forms part of the SCN-autonomic nervous system (ANS). The latter system is involved in resetting the local adrenal clock (local clock). In addition to the actions of the central clock in the SCN, intrinsic mechanisms of the adrenal clock affect the daytime glucocorticoid stimulation by adrenocorticotropic hormone (ACTH), however the influence of this system on the steroidogenic pathway and the resulting cortisol production is of greater significance. The expression of steroidogenic acute regulatory protein (StAR), a rate limiting enzyme in steroidogenesis, is directly influenced by the circadian locomotor output cycles kaput (CLOCK): brain muscle ARNT-like 1(BMAL1) heterodimer which functions as a clock-controlled gene specific to the adrenal gland. The cyclic expression of StAR results in the circadian oscillation in cortisol production through adrenal steroidogenesis (Chung et al., 2011).

HPA axis

Psychological, e.g. stress, and somatic stimuli, e.g. hunger (Ott et al., 2011) and inflammation (Straub et al., 2011), cause the activation of the HPA-axis through the release of CRH, from the paraventricular nucleus (PVN) located in the hypothalamus (Fig. 3.2). After secretion into the hypophyseal portal blood, CRH binds to CRH-binding plasma proteins and is transported to the anterior pituitary where CRH induces the production and secretion of ACTH from proopiomelanocortin (POMC) through sequential cleavage reactions by prohormone convertases PC1/3. In addition to stimulation by CRH, ACTH production and secretion can also be induced by AVP which activates protein kinase C through its effect on a vasopressin 1b (V1b) receptor (Aguilera, 1994). POMC transcription is induced through the activation of adenylate cyclase and results in the production of smaller molecules — pro-ACTH and β-lipoprotein. The subsequent cleavage of pro-ACTH produces ACTH, and two small peptides. It has been suggested that these peptides may contribute to the steroidogenic activity of ACTH (Ackland et al., 1983), although the functions of these peptides are relatively unknown. ACTH binds to the melanocortin type 2 receptor (MCR2), which is expressed in the zona fasciculate and glomerulosa of the adrenal gland, activating the production of cyclic adenosine

monophosphate (cAMP) from ATP through the activation of adenylate cyclase which is induced by the dissociation of G-proteins from the receptor (de Joussineau et al., 2012). The increase in cAMP activates cAMP-dependent protein kinase A (PKA) which phosphorylates targets such as StAR (Glaser and Kiecolt-glaser, 2005). StAR transports cholesterol into the mitochondria for conversion by P450 side-chain cleavage (CYP11A1). The transport of cholesterol into the adrenal mitochondria is the rate limiting step in steroidogenesis as the adrenal steroidogenic enzymes are irreversible and once committed to the pathway, cholesterol will be converted to cortisol and, to a lesser extent, ALDO under ACTH stimulation. In the case of somatic stimuli, the HPA-axis is continuously stimulated, whereas the normal stress response is associated with great variation between subjects with some displaying no resulting elevation of cortisol (Trestman et al., 1991).

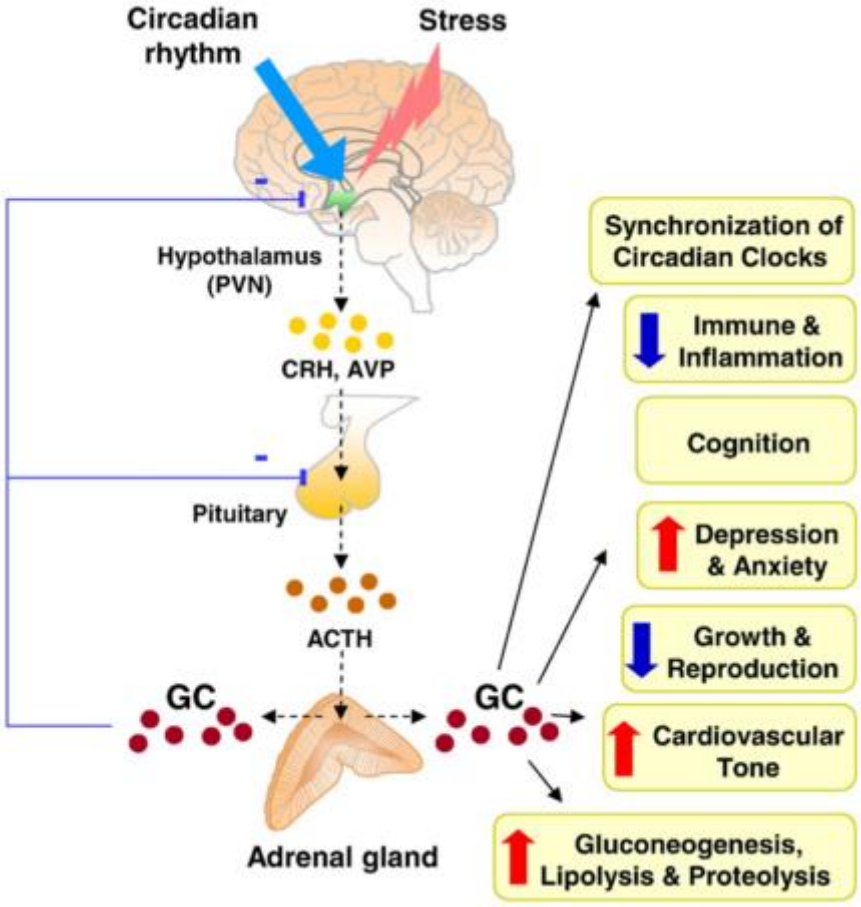

Fig. 3.2: Schematic representation of the regulation of glucocorticoid production by the HPA-axis. (GC, glucocorticoids). Reproduced from Chung et al., 2011.

After the dissipation of the stress stimulus, glucocorticoids inhibit the secretion of CRH and ACTH through feedback inhibition which is mediated primarily through hippocampal GR and MR (Harris et al., 2013). Previous studies have shown that the absence of negative feedback

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by glucocorticoids results in hypersecretion of ACTH and subsequently cortisol (Kadmiel and Cidlowski, 2013). The dysfunction of the inhibition of cortisol production through feedback inhibition of the HPA-axis is associated with the development of chronic stress and inflammation and synthetic glucocorticoids are thus a common therapy for these disorders (Keller et al., 2016; Sigalas et al., 2012).

Under normal physiological conditions, plasma ALDO concentrations are maintained by plasma renin which acts independently from ACTH. Under conditions of stress, ACTH may play a role in increasing ALDO concentrations as described above. Renin is produced in the kidney and is secreted in response to K^+, Ca^{2+}, angiotensin II (AngII) and natriuretic peptides (Spat and Hunyady, 2004). Renin converts angiotensinogen to angiotensin I (AngI), which is converted to AngII through a cleavage reaction catalyzed by angiotensin converting enzyme (ACE). AngII interacts with the AngII receptor Type 1 (AT-1 receptor) to maintain plasma volume and blood pressure by increasing the transcription of aldosterone synthase (CYP11B2) and ultimately, ALDO production. Furthermore, it leads to an increase in the contraction of vascular smooth muscle, catecholamine secretion from the adrenal medulla, enhances sympathetic nervous system (SNS) activities and increases the release of AVP, which in turn may also act on the HPA-axis (Fig. 3.2) (Spat and Hunyady, 2004). AngII causes the depolarization of the cellular membrane and stimulates hormone sensitive lipase and StAR. In addition to the latter mechanism, AngII also induces ALDO secretion by activating the phospholipase C/inositol 1,4,5-triphophate pathway which results in the release of Ca^{2+} ions from the endoplasmic reticulum (ER), activating T-type Ca^{2+} channels (Spät et al., 2016).

The stress response is also associated with the increased secretion of mediators such as norepinephrine (NE), serotonin and acetylcholine. These mediators stimulate cells in the PVN, in the hypothalamus, to produce CRH and subsequent POMC secretion from the anterior pituitary. ACTH, produced from POMC, activates adrenal steroidogenesis and the production of cortisol. During acute stress, growth hormone and glucagon may also act as stress hormones. In this scenario, the inhibitory neurotransmitter, GABA, through its interaction with CRH neurons that act on the PVN, also plays an important role in the regulation of the HPA-axis (Cullinan et al., 2008; Kakizawa et al., 2016). GABA, produced and secreted from neurons, activates neuronal $GABA_A$ ion channels and/or the $GABA_B$ receptor in the plasma membranes. GABA receptor subunits and glutamate decarboxylase (GAD), which catalyses the biosynthesis of GABA, play a prominent role in the GABAergic system. In addition, the expression of both subunits and enzyme has been shown to be similar in other endocrine tissues

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such as the adrenal, testis and ovary (Metzeler et al., 2004). While GABA is the natural ligand, neurosteroids are considered to be allosteric modulators which bind directly to $GABA_A$ receptor and function either in an excitatory or inhibitory manner by affecting the hyperpolarization state of the synapses (Belelli and Lambert, 2005; Gunn et al., 2015; Wang, 2011). These positive/negative allosteric modulatory affects have been shown to be one of the mechanisms through which neurosteroids affect rapid, non-genomic effects that play an important role in the stress response (Chisari et al., 2010; Zorumski et al., 2013).

Steroid biosynthesis

Corticosteroid biosynthesis occurs mainly within the adrenal gland and these steroids are associated mostly with genomic effects, while other corticosteroid metabolites are biosynthesized in the central nervous system tissues (brain and spinal cord) where they are associated with rapid, non-genomic effects (Strömberg et al., 2005). As both these mechanisms are important in describing the overall peripheral effects of corticosteroids, the biosynthesis of adrenal and neurosteroids will be discussed below. However, as the main focus of this study is adrenal steroidogenesis, the biosynthesis of neurosteroids in the brain will be discussed only in terms of their effects on neurotransmission via GABA receptors which is known to affect the HPA-axis.

Adrenal steroidogenesis

Adrenal steroidogenesis can be divided into three major branches: 1) glucocorticoid production which occurs in the zona fasciculata, 2) mineralocorticoid production which occurs in the zona glomerulosa and 3) androgen production which occurs in the zona reticularis (Fig 3.3) (Vinson, 2003). Adrenal steroidogenic enzymes are usually unidirectional and do therefore not allow steroid flux to drive precursor production when steroid hormone levels are increased. The hydroxylation reactions catalyzed by the P450 enzymes are thus irreversible, however, some HSD catalyzed reactions are essentially reversible in an *in vitro* environment (Miller and Auchus, 2011).

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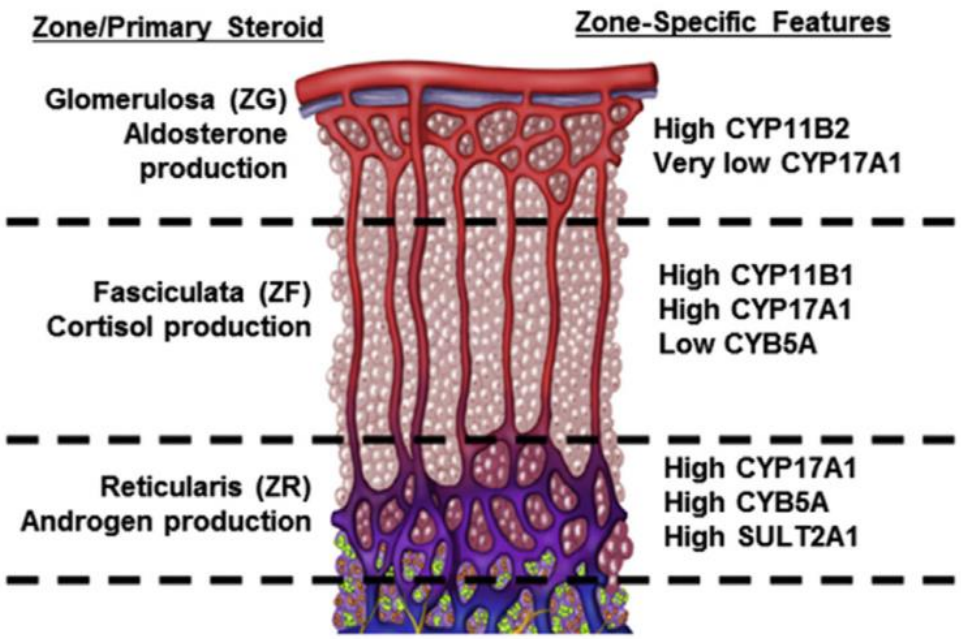

Fig. 3.3: Expression patterns of steroidogenic enzymes in the adrenal gland. Reproduced from Turcu and Auchus, 2016.

Adrenal P450 enzymes catalyze the biosynthesis of the steroid hormones in the mineralocorticoid, glucocorticoid and androgen pathways (Fig 3.4). These steroids are all derived from the precursor steroid, cholesterol, with CYP11A1 catalyzing its conversion to PREG. In the mineralocorticoid pathway, limited to the zona glomerulosa of the adrenal cortex, PREG is converted by 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2) to PROG and subsequently to deoxycorticosterone (DOC) by CYP21A2. DOC is converted to ALDO via CORT and 18-hydroxycorticosterone (18OH-CORT) by CYP11B2. In the glucocorticoid pathway, 17OH-PREG, a product of the CYP17A1 conversion of PREG, is converted to cortisol via 17 hydroxy-PROG (17OH-PROG) and deoxycortisol, catalyzed by 3β-HSD2, CYP21A2 and P450 11β-hydroxylase (CYP11B1) respectively. In the androgen pathway, dehydroepiandrosterone (DHEA), the product of 17OH-PREG, is converted to androstenedione (A4) by 3β-HSD2 (Miller and Auchus, 2011; Payne and Hales, 2004). We recently showed that A4 is also a substrate for CYP11B1 which catalyzes the formation of 11-hydroxyandrostenedione (11OHA4) from A4 (Swart et al., 2013).

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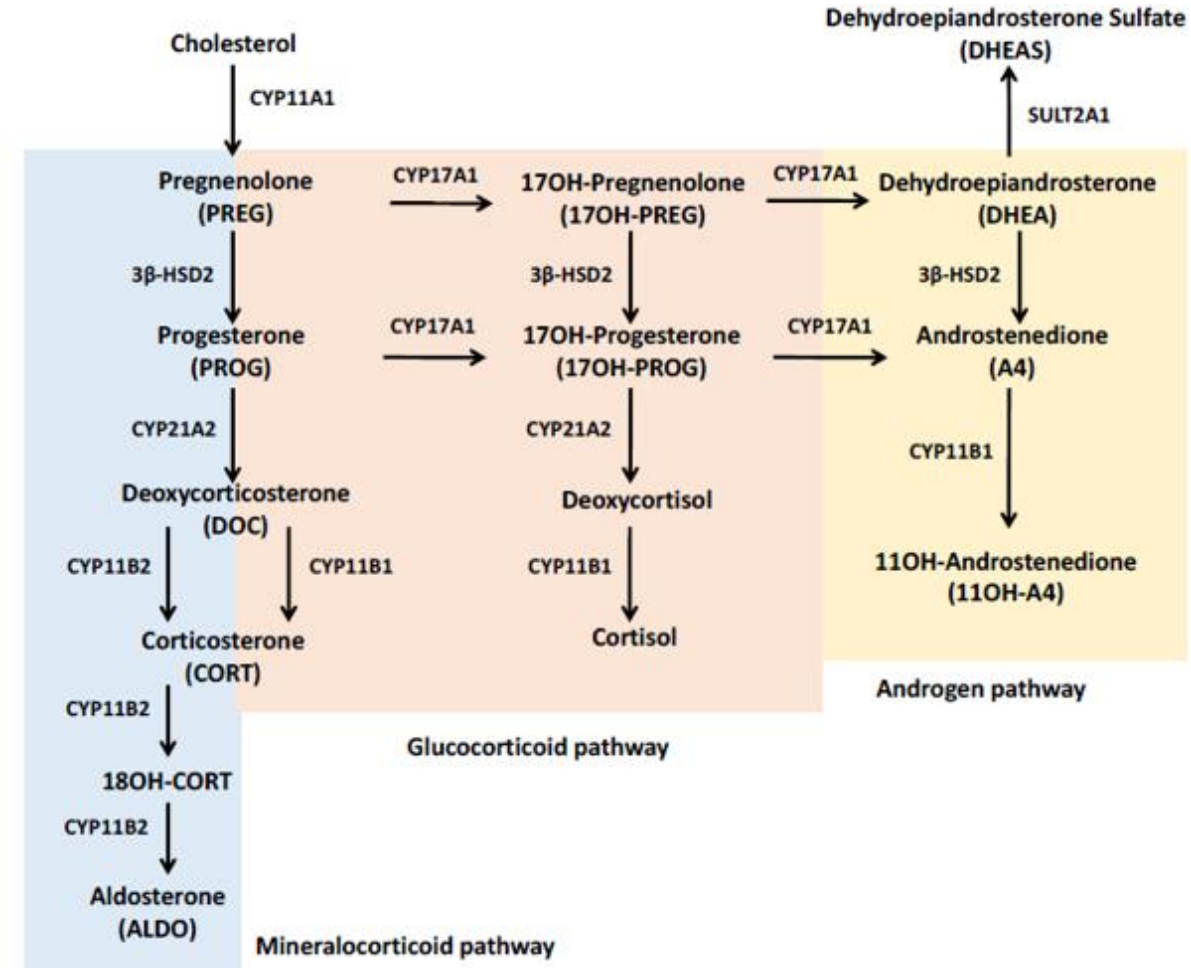

Fig. 3.4: Human adrenal steroid biosynthesis. The metabolism of cholesterol by the major steroidogenic enzymes yielding ALDO, cortisol and 11OH-A4 in the mineralocorticoid- glucocorticoid- and androgen pathways, respectively.

Neurosteroid biosynthesis in the brain

In addition to steroids produced in endocrine tissues, several PREG and DOC metabolites are biosynthesized in the brain (Paul and Purdy, 1992). These steroid metabolites, termed neurosteroids, elicit diverse effects pertaining to stress, mood, behavioral changes, sedation and anxiety (Zorumski et al., 2013). It was originally assumed that the steroids eliciting the aforementioned effects were produced in the adrenal (or other endocrine tissues) and that no steroid biosynthesis occurred in the brain. However, CYP11A1, CYP17A1, 3β-HSD2, CYP21A2, 11β-HSD, GR and MR mRNA have been detected in the human amygdala, cerebellum, spinal cord and hippocampus. In contrast, CYP11B1 mRNA was not detected in the cerebellum or hippocampus, and it has been suggested that glucocorticoid related effects were dependent on circulatory glucocorticoids (Yu et al., 2002). Although studies have shown that ALDO is produced in the brain, these studies showed that corticosteroid production in the

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brain is very low and thus may not contribute significantly to circulating corticosteroids levels. Therefore, the effects elicited by the corticosteroids in the brain are dependent on corticosteroid production in other endocrine tissues with the majority of corticosteroids produced in the adrenal (glucocorticoids and mineralocorticoids (Gomez-Sanchez et al., 2005).

As is the case in adrenal steroidogenesis, cholesterol is the precursor for steroid biosynthesis in the brain. After being transported into the inner mitochondrial membrane by StAR, cholesterol is converted to PREG by CYP11A1. PREG is subsequently converted to PROG by 3β-HSD2, where after PROG and DOC, produced from PROG by CYP21A2, are reduced to their 5α-metabolites by 5α-reductase (Fig. 3.5). The 5α-reduced products, 5α-dihydroprogesterone (5α-DHPROG) and 5α-dihydrodeoxycorticosterone (5α-DHDOC), are converted to the neuroactive steroids *viz*. 3α,5α-tetrahydroprogesterone (3α,5α-THPROG) and 3α,5α-tetrahydrodeoxycorticosterone (3α,5α-THDOC), by 3α-hydroxysteroid oxidoreductase (Rupprecht and Holsboer, 1999). These steroids elicit effects via $GABA_A$ receptors by acting as GABA agonists in neurotransmission (Weng and Chung, 2016). PREG is also converted to DHEA via the typical adrenal steroidogenic reactions (Fig.3.4) and their sulfonated conjugates, PREG sulfate (PREGS) and DHEA sulfate (DHEAS), produced by Sulfotransferase (SULT) 2A1, elicit antagonistic effects towards the $GABA_A$ receptor (Rupprecht, 2003). Downstream sex hormones, such as testosterone and estradiol, have also been shown to modulate $GABA_A$ receptor activity with increased testosterone levels associated with adverse behavioral changes such as violence (Miczek et al., 2003).

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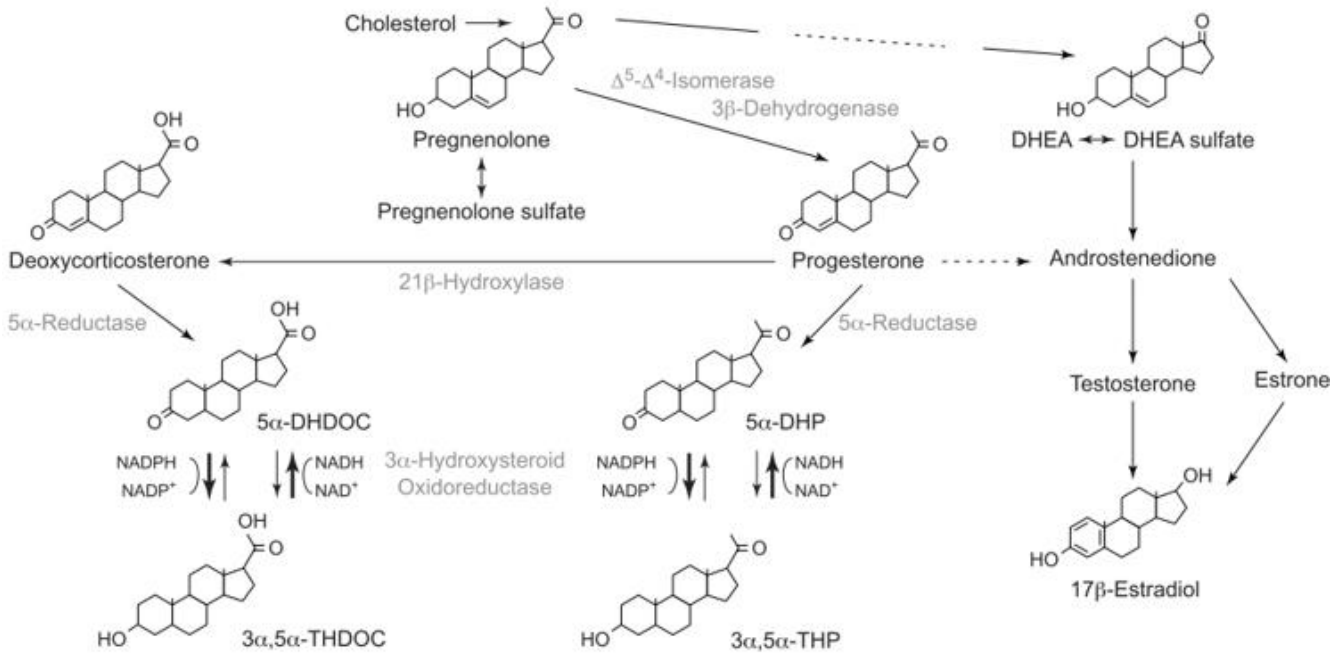

Fig. 3.5: Biosynthesis and metabolism of neuro-active steroids. The dotted lines represent intermediates not shown. 5α-DHDOC, dihydrodeoxycorticosterone; 5α-DHP, dihydroprogesterone; 3α,5α-THDOC, tetrahydrodeoxycortico-sterone; 3α,5α-THP, tetrahydroprogesterone. Reproduced from Rupprecht and Holsboer, 1999.

Overview of cytochrome P450 enzymes

P450 enzymes were named after the ability of these enzymes, in their reduced states, to absorb light at 450 nm. These membrane bound enzymes are oxidative enzymes which contain ± 500 amino acids and a heme group with conserved genetic sequences in all 57 *CYP* genes identified in the human body (Lander et al., 2001). Six P450 enzymes are involved in adrenal steroidogenesis, however, only the enzymes depicted in Fig 3.4 will be discussed as these enzymes are relevant to this study. CYP11A1, CYP11B1 and CYP11B2 are associated with the mitochondrial membrane (Type 1 enzymes), whereas CYP17A1 and CYP21A2 are associated with the ER (Type II) (Miller, 2005). In addition to the adrenal P450 enzymes, 3β-HSD2 and SULT2A1 together with low levels of 11β-HSD and 17β-HSD3 and 17β-HSD5, are also expressed in the adrenal gland and are involved in adrenal steroidogenesis.

Active site structure and substrate binding

The P450 enzyme family share approximately 20% sequence identity and a similar general protein fold (Hasemann et al., 1995). The enzyme's structural core consists of four helices — three of which are arranged in parallel (helices D, L and I) and the other, antiparallel (helix E). The heme, bound in the Cys-heme-ligand loop, is located between helices I and L. The Cys-heme-ligand loop contains the conserved P450 amino acid sequence (FxxGx(H/R)xCxG) of

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which Cys is conserved throughout P450 enzymes. The Cys residue is responsible for the characteristic Soret peak observed at 450 nm when the reduced enzyme is bound to carbon monoxide (Omura and Sato, 1964). Cys characteristically binds to backbone amides through hydrogen bonding, while the I helix, containing the (A/G)Gx(E/D)T amino acid sequence in the middle of the helix, constitutes the wall of the heme pocket. The Thr residue, which is highly conserved, is nestled in the active site and it has been suggested that it is involved in the catalytic activity of the enzyme (Imai et al., 1989). In spite of the highly conserved protein fold of the P450 enzymes, enough diversity exists to allow for the binding of structurally diverse substrates. Although this is not the case for adrenal P450 enzymes that are highly specific towards their substrates, CYP3A4 for example can metabolize xenobiotics and commercial drugs displaying diverse substrate binding abilities. In addition, it has also been shown that P450 enzymes may bind to more than one substrate at a time (Korzekwa et al., 1998).

Substrate binding to P450 enzymes is facilitated by six substrate recognition sites (SRS), as depicted in Fig. 3.6. The SRSs are SRS1 within the B-helix, SRS2 and SRS3, which comprise parts of the F and G helices, SRS4 in the I helix, SRS5 which constitutes the β4 hairpin and SRS6 comprised of the connecting region of the K helix. All of these SRSs line the inside of the P450 active pocket, influencing substrate specificity while retaining flexibility within the regions to accommodate an induced-fit binding mechanism. The latter mechanism favours substrate binding and results in catalytic activity of the enzyme towards the substrate. Point mutations within the SRS have been shown to affect substrate binding significantly, potentially resulting in endocrine disorders (Gotoh, 1992; Pylypenko and Schlichting, 2004).

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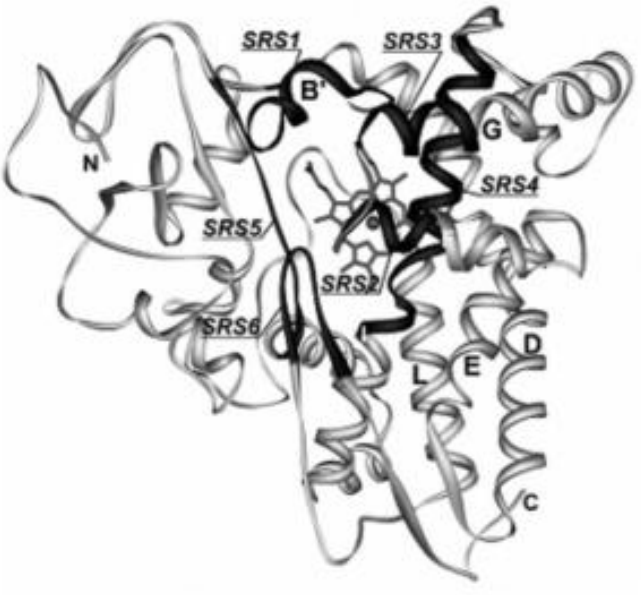

Fig. 3.6: Ribbon representation of the conserved P450 fold depicting SRSs and helices labeled as discussed in the text. Reproduced from Denisov et al., 2005.

Catalytic cycle

The catalytic cycle, common to all P450 enzymes, comprises the two-electron reduction and the formation of oxygenated intermediates (Estabrook et al., 1971) and is depicted in Fig. 3.7. After substrate binding (reaction 1, Fig. 3.7), the redox potential of the heme is increased (-300 mV to -170 mV) as a result of a conformational change being induced in the enzyme. Electrons are transferred from the reduced enzyme-substrate complex after substrate binding, resulting in the increased affinity of the enzyme for oxygen as the free enzyme has very low affinity for oxygen. One oxygen molecule subsequently reduces the heme at the 6th axial position, causing the bound electron to be transferred to the bound oxygen molecule. The resulting oxygenated P450 enzyme-complex is highly unstable and requires the transfer of the second electron by the electron carrier. At this step, some microsomal P450 enzymes may acquire the electron from cytochrome b_5, although this reaction is species specific. The heme-bound oxygen subsequently reacts with the protons which result in the release of H_2O from the active site, and an activated oxygen molecule which reacts with the substrate in the substrate-enzyme complex where after the product is released. The overall reaction is represented by equation 4.1 (Coon, 2005; Denisov et al., 2005; Werck-reichhart and Feyereisen, 2000).

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \qquad \text{Equation 4.1}$$

Studies in liver microsomes showed that molecules more hydrophobic in nature are more likely to bind to the active site of the P450 enzymes and the affinity of the P450 enzymes for a

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particular substrate increases with a decrease in hydrophilicity. This phenomenon could be attributed to the hydrophobic nature of the enzyme active pocket due to the hydrophobic amino acid residues located in the pocket. The conformational changes induced by substrate binding ensures appropriate substrate alignment to the activated oxygen molecule. The conformational changes induced by the binding of the substrate and the hydrophobicity of the active pocket relate to the stereospecificity of P450 enzymes (Xu et al., 2010).

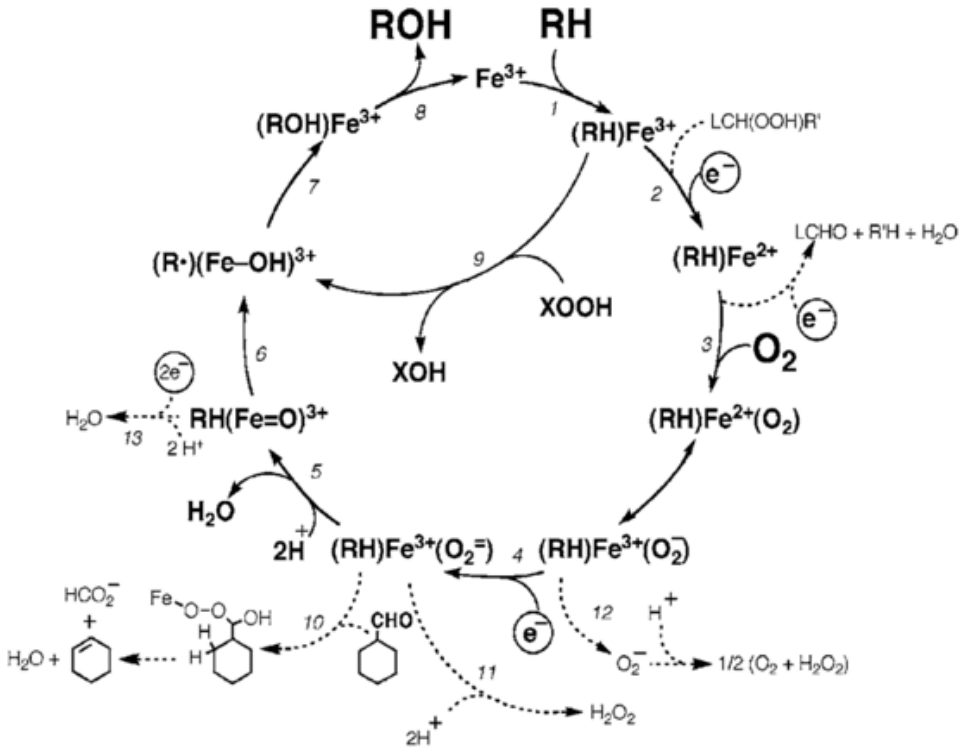

Fig. 3.7: Catalytic cycle of P450 enzymes. RH represents the steroid substrate (or drug in the case of hepatic P450 enzymes) and ROH represents the steroid product formed. Reproduced from Coon, 2005.

Electron transfer

The transfer of electrons is of critical importance in the catalytic cycle of P450 enzymes as described above. There are however two mechanisms though which electron transport occurs, depending on the location of the enzyme. Fig. 3.8A shows the electron transport from electron carriers to the mitochondrial P450 enzymes — CYP11A1, CYP11B1 and CYP11B2. In mitochondria the flavin redox cofactor, flavin adenine dinucleotide (FAD), of ferrodoxin reductase (FeRed), also known as adrenodoxin reductase, is bound to the inner membrane where it gains two electrons from NADPH, reducing it to $NADP^+$. The iron-sulphur cluster of ferrodoxin (Fedx), also known as adrenodoxin, accepts the electrons and subsequently transfers

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the electrons to the P450 heme. The transfer of the electrons is guided by the negatively and positively charged residues present in Fedx (-) and FeRed (+) and the P450 (+) enzymes. In the case of CYP11A1, three electron pairs are required for the conversion of cholesterol to PREG (Miller and Auchus, 2011; Papadopoulos and Miller, 2012).

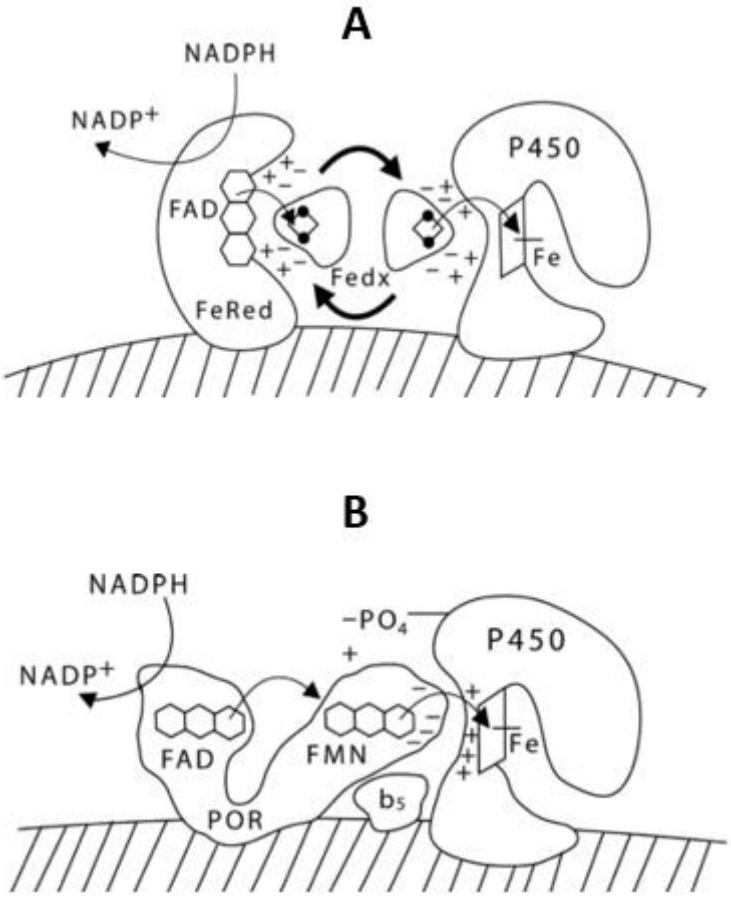

Fig. 3.8: Electron transfers mechanisms employed by A) mitochondrial and B) microsomal P450 enzymes. Reproduced from Miller and Auchus, 2011.

The electron transfer system utilized by microsomal P450 enzymes is depicted in Fig. 3.8B. P450 oxidoreductase (POR), bound to the ER, accepts the electron pair from NADPH which is transferred to the FAD moiety of POR. The conformational change induced by the electron gain rearranges the FAD and flavin mononucleotide (FMN) moieties so that their isoalloxazine ring structures are in close proximity, allowing the transfer of the electron from FAD to FMN. The 2nd electron transfer induces another conformational change, returning the protein to its original conformation while the FMN moiety of POR reacts with the appropriate binding site of the P450 enzyme, mediating catalysis through interaction with the P450 heme. The interaction between the FMN moiety of POR and the redox-binding site of P450 is mediated by the negatively charged residues on the FMN moiety and the positively charged residues in

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the P450 binding site — an interaction similar to the one observed between Fedx and the mitochondrial P450 enzymes. The steroid substrate is bound to the enzyme on the side of the heme ring located opposite to the FMN moiety. This interaction is modified in human CYP17A1 as the binding of the steroid in the active pocket of the enzyme is facilitated by the allosteric influence of cytochrome b_5 (Lee-Robichaud et al., 1995; Miller and Auchus, 2011).

Spectral properties

P450 enzymes were discovered when an unusual absorption spectrum at 450 nm was observed when assaying the redox kinetics of cytochrome b_5 in liver microsomes. Absorption was observed at 450 nm after carbon monoxide saturation of the sample and as carbon monoxide saturation does not affect α and β bands of cytochrome b_5, investigators could not attribute the phenomenon to the presence of cytochrome b_5. The results suggested that the pigment contained a heavy metal ion due to saturation by carbon monoxide, however, it did not resemble any of the known hemo or metalloproteins (Garfinkel, 1958; Klingenberg, 1958).

Researchers speculated that this new pigment was a cytochrome b_5 isoform and named it P450 (Omura and Sato, 1964). When reduced with ethyl isocyanide, the pigment displayed a difference spectrum characteristic of a hemoprotein showing intense bands in the Soret region. Results also showed that the treatment of microsomes with detergents did not obliterate the pigment and allowed it to bind to carbon monoxide and remain spectrally independent in its reduced form. After solubilization, the pigment displayed a distinctly different spectrum after carbon monoxide saturation displaying a peak at 420 nm, subsequently referred to as the P420 and confirmed the hemoprotein nature of the pigment.

The spectral data was used to calculate an extinction coefficient (91 $cm^{-1}mM^{-1}$) for the carbon monoxide reduced-difference spectrum of the pigment (420 – 490 nm) (Omura and Sato, 1964), which was used to determine the concentration of P450 enzymes present in microsomes. The method has since been used to determine the P450 enzyme content of many other tissues and was also applied in the current study.

As previously discussed, the binding of substrate to a P450 enzyme induces a conformational change associated with a change in spin state. The enzyme-substrate complex displays an absorbance maximum at 391 nm when in the high spin state and while the absorbance maximum of reduced enzyme-substrate complex shifts to 408 nm, the reaction with molecular oxygen shifts the absorbance maxima to 418 nm. The observed shifts in maximum absorbance and the resulting spectra are a result of the energy level of the heme locus which may be

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affected by the environment of the heme-locus and the hydrophilicity of the heme-ligand, as well as the bound substrate or inhibitor (Kominami and Takemori, 1982). The binding of substrates to P450 enzymes results in difference spectra — Type I, II (Fig 3.9) and reverse Type I were identified depending on the occupation of the P450 enzyme active site.

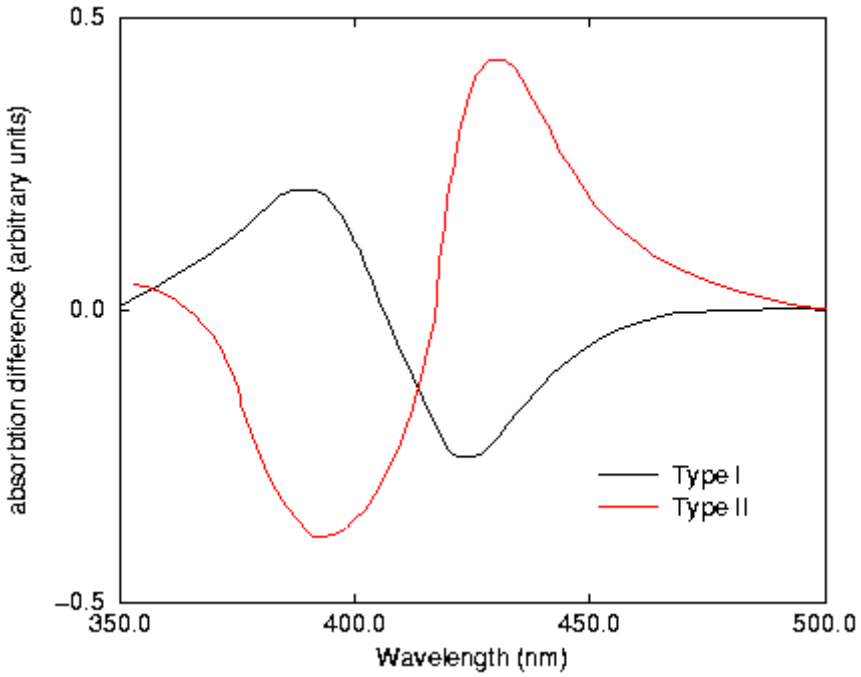

Fig. 3.9: Type I and II difference spectra obtained after the binding of a substrate or inhibitor to P450 enzymes visualized under UV light. Reproduced from Omura et al., 1993.

Type I difference spectra typically represents the interaction between the enzyme and its substrate, displaying a maximum and a minimum peak at 390 and 420 nm, respectively. The Type I substrate characteristically shifts the heme spin state equilibrium from a low to a high spin state resulting in the displacement of a water molecule and an iron shift to the thiolate ligand from the planar porphyrin. The majority of P450 substrates are classified as Type I substrates and spectral analyses is utilized to determine the amount of bound vs unbound enzyme, i.e. the amount of high spin vs low spin enzyme (Kumaki et al., 1978; Omura et al., 1993).

Type II difference spectra absorb maximally in the range of 425-435 nm and minimally between 390-405 nm. These spectra are usually observed in the presence of inhibitors of P450 enzymes and can be induced in the presence of nitrogen-rich, sulphur or oxygen containing compounds, as well as compounds with unbound electrons (Kumaki et al., 1978; Omura and Sato, 1964).

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Reverse Type I spectra are characteristically induced by compounds that bind to sites other than the active site of the P450 enzymes, absorbing maximally in the range of 409-445 nm and minimally between 365-410 nm. These spectra are usually induced by lipophilic compounds that cause the displacement of the water molecule after substrate binding has occurred. Studies have shown that the majority of compounds which elicit a reverse type I spectrum display competitive inhibition characteristics and follow typical Michealis-Menton saturation kinetics, however some isoforms have been shown to display non-hyperbolic kinetics (Hlavica and Lewis, 2001).

Adrenal steroidogenic enzymes

Adrenal steroidogenesis, with particular focus on corticosteroid production, typically involves five P450 enzymes and 3β-HSD2. Although 11β-HSD1 and 11β-HSD2 are typically expressed in peripheral tissue with only low levels detected in the adrenal gland, these enzymes play a critical role at the target sites of glucocorticoids and mineralocorticoids and will be discussed in this section. Additionally, SULT2A1 will be discussed as it is expressed in the adrenal and in the adrenal cell model that was utilized in this study.

CYP11A1

CYP11A1, a single, 20 kb gene product, catalyzes the conversion of cholesterol to PREG and isocaproaldehyde in the rate limiting step of steroidogenesis (Fig. 3.10). The conversion of cholesterol constitutes three sequential oxidation reactions, requiring a total of three oxygen and three NADPH molecules. The reaction is facilitated by the mitochondrial electron transfer system utilizing FeRed as previously described in this chapter. Cholesterol undergoes hydroxylation at C22 with the subsequent hydroxylation at C20 to produce 20,22R-hydroxycholesterol. The latter steroid intermediate is cleaved between C20 and C22 to produce PREG and isocaproaldehyde, which is ultimately converted to isocaproic acid (Burstein and Gut, 1976; Teicher et al., 1978).

CYP11A1 is expressed in all three zones of the adrenal cortex, as well as in other steroidogenic tissues such as the placenta, testis and ovary. In addition, studies have shown that CYP11A1 is expressed in skin, in the nervous system, peripheral and central, as well as cardiac tissues (human and rodent) (Pelletier et al., 2001).

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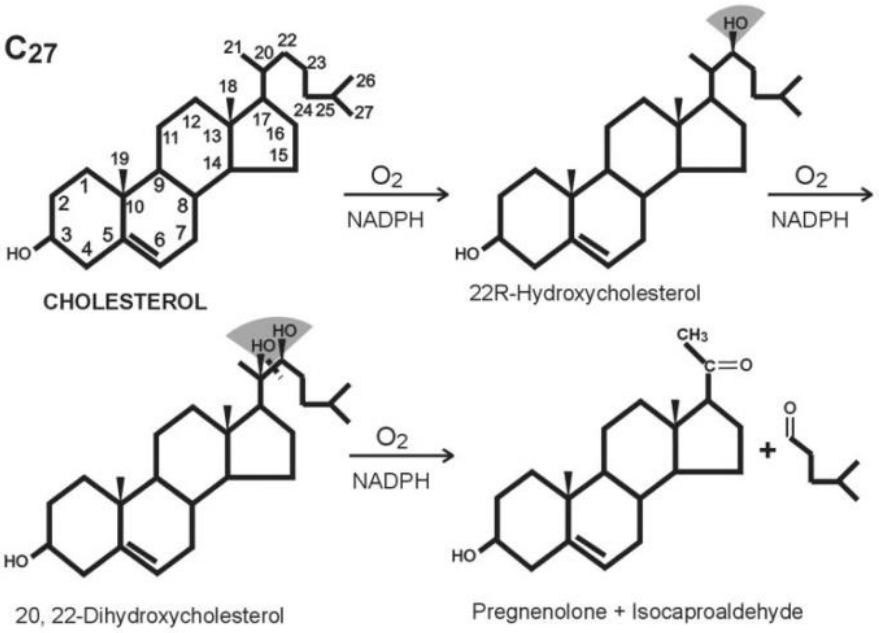

Fig. 3.10: Reactions catalyzed by CYP11A1 in the mitochondrial membrane. Cholesterol is converted to PREG via three sequential oxidation reactions, each utilizing one molecule of NADPH and one molecule of oxygen. The six carbon side chain is cleaved during the final oxidation reaction yielding isocaproaldehyde and PREG. Reproduced from Payne and Hales, 2004.

CYP17A1

CYP17A1, together with 3β-HSD2, represents a significant branch point within adrenal steroidogenesis, ultimately influencing the steroid flux in the glucocorticoid-, mineralocorticoid- and androgen pathways. CYP17A1 is associated with the ER while utilizing the microsomal electron transfer system for electron transport in which it catalyses the conversion of more than one substrate. CYP17A1 catalyzes the 17α-hydroxylation and lyase reaction of PREG, requiring one molecule of oxygen and one molecule of NADPH for each reaction (as shown in Fig.3.11) to form 17 hydroxy-PREG (17OH-PREG) which is subsequently cleaved at the C20-C17 bond to produce DHEA. CYP17A1 also catalyzes the 17α-hydroxylation of PROG yielding 17OH-PROG, however, in humans the lyase reaction does not progress to A4. The lyase activity is not present in all species and it has been found that for example, both human and bovine CYP17A1 lyase activity displays preference for 17OH-PREG as substrate leading to the production of DHEA, whereas the rodent enzyme shows preference for 17OH-PROG leading to A4 production. This species specific variation in lyase activity has been attributed, in part, to human and bovine species requiring cytochrome b_5 for the promotion of the lyase activity (Lee-Robichaud et al., 1995) and has been associated with post-translational modifications of the enzymes (Miller and Tee, 2014). Rat CYP17A1,

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not expressed in the adrenal cortex (Pelletier et al., 2001), also requires cytochrome b_5 for its lyase activity, but the fold change induced is less than that observed in bovine and human species. The lyase activity of CYP17A1 is regulated by the availability of electrons for this reaction and is thus regulated by cytochrome b_5, high concentrations of POR and serine phosphorylation of the enzyme (Suzuki et al., 2000).

Human CYP17A1 also converts PROG to 16 hydroxy-PROG (16OH-PROG), usually in a 17OH-PROG:16OH-PROG ratio of 3:1, while this was not observed for baboon CYP17A1 (Swart et al., 2002). The ability of CYP17A1 to produce 16OH-PROG from PROG has been attributed to the presence of an alanine residue at position 105 in the human protein, rather than the leucine residue present in other species. 16OH-PROG is not metabolized further in the adrenal and significant concentrations have been detected in plasma and urine samples. The function of this steroid intermediate has been linked to antagonistic effects, competing with PROG, testosterone and β-estradiol at their target tissues and it has been found to act as an agonist of the human PROG receptor (PR) (Storbeck et al., 2011).

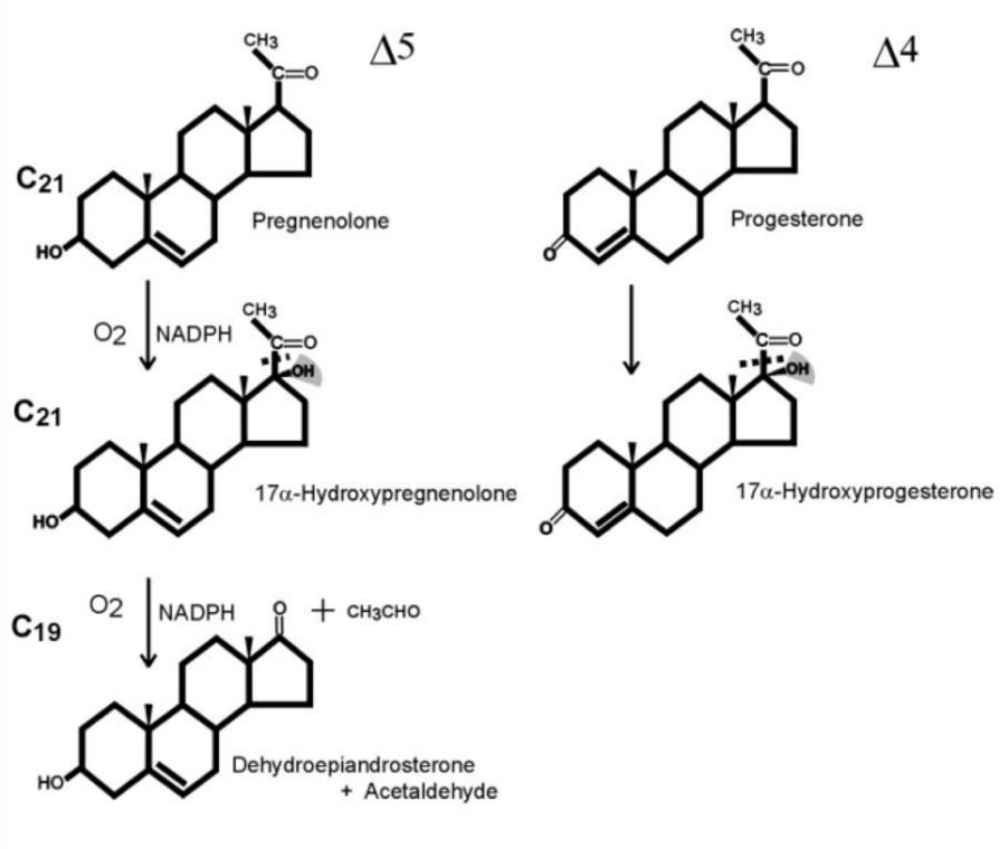

Fig. 3.11: CYP17A1 catalyzes the conversion of PREG to DHEA and PROG to 17OHPROG through two mixed function oxidase reactions. One molecule of oxygen and one molecule of NADPH is utilized for each of the hydroxylation and cleavage reactions while electrons are

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supplied by the microsomal electron transfer system. Reproduced and adapted from Payne and Hales, 2004.

3β-HSD2

The HSDs catalyze the reduction and oxidation reactions of steroid hormones and require NADH or NADPH as donors and NAD^+/$NADP^+$ as electron acceptors of reducing equivalents. HSDs differ from P450 enzymes with regard to the fact that HSDs are often represented by multiple isoforms, whereas P450 enzymes are transcribed from one distinct gene.

3β-HSD/isomerases are bound to the membranes of mitochondria and ER, depending on the cell type in which they are expressed (Pelletier et al., 2001). Multiple isoforms of this enzyme exists in various species, with the highest number of isoforms identified in mice (Payne et al., 1997). In humans, two distinct isoforms have been identified — 3β-HSD1 and 3β-HSD2, both of which have dehydrogenase/isomerase activity. The reactions catalyzed by 3β-HSD are critical for the production of steroid levels, together with CYP17A1, in the three steroidogenic pathways in the adrenal with the steroid flux also depending on the catalytic activity of 3β-HSD (Goosen et al., 2010). 3β-HSD catalyzes the conversion of PREG to PROG, 17OH-PREG to 17OH-PROG and DHEA to A4 by sequential dehydrogenase and isomerase reactions (Fig 3.12) (Thomas et al., 1995). The dehydrogenation reaction reduces NAD^+ to NADH and results in the formation of a $\Delta^{5\text{-}3}$ keto-steroid intermediate which remains bound to the enzyme. NADH, also bound to the enzyme, induces the isomerization of the intermediate through conformational changes and produces the Δ^4-steroids (Thomas et al., 1995). Although cytochrome b_5 has not been shown to bind to 3β-HSD directly, studies have shown that cytochrome b_5 augments 3β-HSD's activity allosterically by increasing the affinity of the enzyme towards NAD^+ (Goosen et al., 2013). In addition, human and murine 3β-HSD has been shown to convert A4 to dihydrotestosterone while reducing NAD^+. $NADP^+$ has not been associated with the latter reaction catalyzed by 3β-HSD (Clarke et al., 1993).

Although 3β-HSD2 is considered to be one of the major branch point enzymes in steroidogenesis, associated with the regulation of cortisol production, recent evidence suggests that another isoform, 3β-HSD1, plays a major role in ALDO production. However, 3β-HSD1 is expressed at much lower levels that 3β-HSD2 (Konosu-Fukaya et al., 2014).

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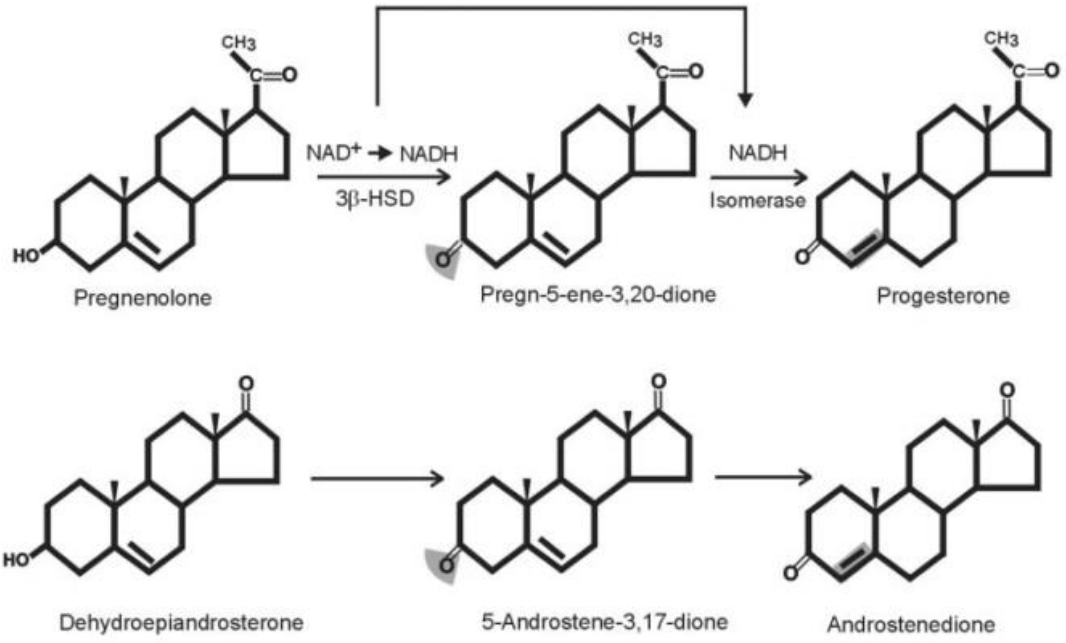

Fig. 3.12: 3β-HSD catalyzes sequential dehydrogenization and isomerization reactions converting PREG to PROG and DHEA to A4. Reproduced from Payne and Hales, 2004.

SULT2A1

Steroid sulfates are derived either directly from cholesterol or through the sulfonation of steroids (Strott, 2002). The latter reaction is catalyzed by the SULT enzymes and although there are approximately 44 isoforms of this enzyme, only SULT2A1 will be discussed for the purposes of this study.

SULT2A1, the major SULT in the adrenal gland, sulfonates PREG, 17OH-PREG, DHEA and A4, but not cholesterol directly. The reaction catalyzed by the SULT enzymes utilizes 3'-phosphoadenosine-5'-phosphosulfate (PAPS), which is synthesized from ATP, as sulfate donor. PAPS is synthesized from the conversion of ATP and sulfate to adenosine phosphosulfate (APS) by ATP sulfurylase, which is subsequently converted from APS to PAPS by APS kinase using the phosphate from another ATP molecule (Strott, 2002). In humans, the latter two reactions are catalyzed by one enzyme either ubiquitously expressed, PAP synthase type 1 (PASS1), or expressed in the major sites of DHEA sulfonation, PAP synthase type 2 (PASS2), which is in the liver and adrenal glands. Inhibition of DHEA sulfonation by PAPSS2 leads to an increase in free DHEA which can be converted to adrenal androgens. The perturbation of the DHEA:DHEAS ratio has been shown to correlate with an increased risk of cancers, especially prostate cancer (Nowell and Falany, 2006).

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CYP21A2

CYP21A2 is expressed in all three zones of the adrenal cortex (Parker et al., 1985) and is essential for the production of cortisol, the major glucocorticoid, and ALDO, the major mineralocorticoid. The enzyme catalyzes the conversion of PROG to DOC and 17OH-PROG to deoxycortisol. As CYP21A2 is located in the ER, it employs the microsomal electron transfer system requiring one molecule of oxygen and NADPH (Fig 3.13).

In the zona glomerulosa, PROG serves as the substrate for CYP21A2. As CYP17A1 is not expressed in this zone, only DOC is produced as no 17OH-PROG is produced for conversion to deoxycortisol and ultimately ALDO production takes preference in this zone (Rice et al., 1990).

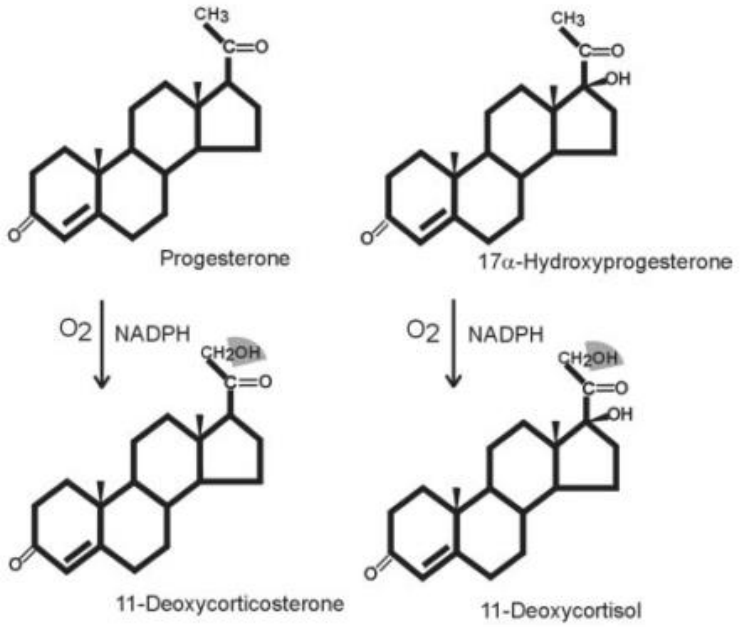

Fig. 3.13: CYP21A2 catalyzes the conversion of PROG to DOC and 17OH-PROG to deoxycortisol by C21 hydroxylation which requires one molecule of oxygen and one molecule of NADPH. Reproduced fom Payne and Hales, 2004.

CYP11B1 and CYP11B2

CYP11B1 and CYP11B2 are positioned in the inner membrane of the mitochondria and catalyze the final reactions in glucocorticoid and mineralocorticoid production. CYP11B1 converts deoxycortisol and DOC to cortisol and CORT, respectively, through an 11β-hydroxylation reaction (Fig 3.14), whereas CYP11B2 converts DOC to ALDO through three sequential reactions involving CORT and 18OH-CORT as intermediates (Fig 3.15). It has been suggested that the conversion of DOC to ALDO by CYP11B2 occurs without the release of

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the intermediates, much like the conversion of cholesterol to PREG by CYP11A1 (Kawamoto et al., 1992). However, these intermediates can be detected in adrenal cell models such as H295R cells (Schloms et al., 2012). Furthermore, CORT is not utilized by CYP11B2 in the production of ALDO confirming that ALDO is produced though the conversion of DOC and the reaction is catalyzed by CYP11B2 only (Kawamoto et al., 1992). Three CYP11B isoforms are expressed in the rat adrenal with the B1 and 2 isoform catalyzing reactions as described above (with the exception of cortisol production due to the lack of CYP17A1 expression) and B3 producing 18OH-CORT from DOC, but lacking the 18-oxidase activity (Mellon et al., 1995).

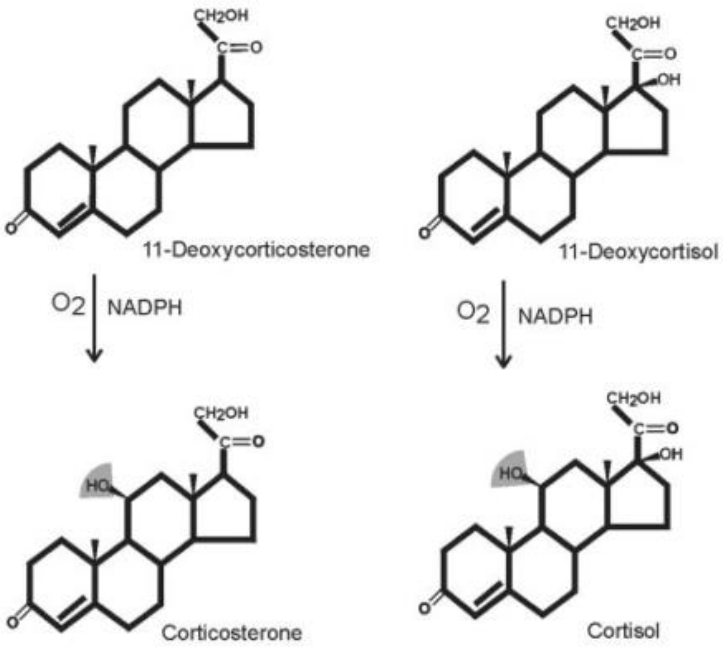

Fig. 3.14: CYP11B1 catalyzes the conversion of DOC to CORT and deoxycortisol to cortisol by 11β-hydroxylation, which requires one molecule of oxygen and one molecule of NADPH. Reproduced from Payne and Hales, 2004.

CYP11B1 and *CYP11B2* are closely linked and found on the same human chromosome approximately 40 kb apart (Lifton et al., 1992). CYP11B1 and CYP11B2 are expressed only in the adrenal cortex with CYP11B1 expressed in the zona fasciculate and reticularis (Curnow et al., 1991). CYP11B2, although expressed to a much lesser extent than CYP11B1, is expressed exclusively in the mitochondria of the zona glomerulosa (Domalik et al., 1991).

Recent investigations have provided evidence for the involvement of CYP11B1 in the conversion of A4 to 11OHA4, while CYP11B2 showed no effect on 11OHA4 production. In addition, testosterone was significantly hydroxylated by CYP11B1 and CYP11B2, however

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the production of 11β-hydroxytestosterone (11OHT) was not as efficient as the production of 11OHA4 by CYP11B1 (Swart et al., 2013).

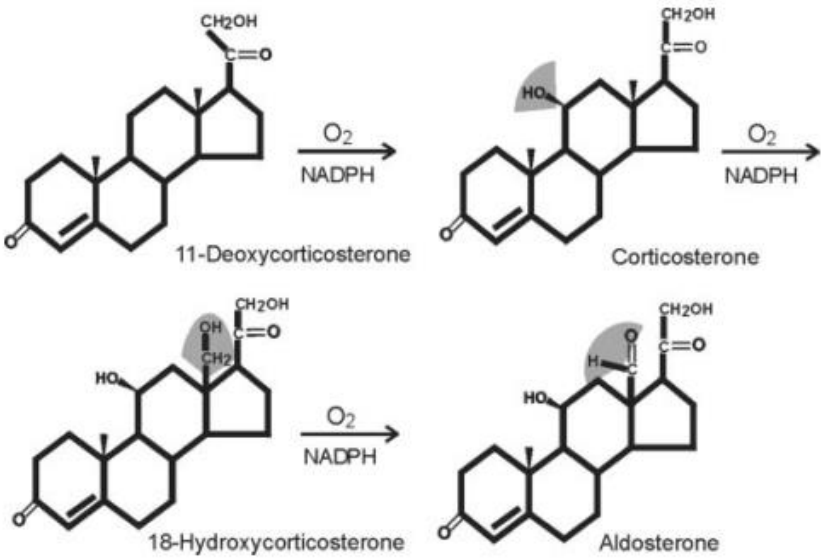

Fig. 3.15: CYP11B2 catalyzes the conversion of DOC to ALDO via three sequential reactions utilizing one molecule of oxygen and one molecule of NADPH per reaction. Reproduced from Payne and Hales, 2004.

11β-HSD1 and 11β-HSD2

11β-HSD enzymes are commonly co-expressed with the MR in glucocorticoid responsive tissues, such as the kidney, since these enzymes catalyze the interconversion of cortisol and cortisone, eliciting an MR-protecting function, as well as CORT and dehydrocorticosterone (11-DHC) (Fig. 3.16). Studies have shown that the MR has equal affinities for cortisol and ALDO (Arriza et al., 1987) and as plasma cortisol concentrations are generally 100 to 1000-fold greater than that of ALDO, cortisol is converted to its inactive form, cortisone, by 11β-HSD2 (Funder, 2005; Funder et al., 1988). The ability of 11β-HSD1 to either catalyze the oxidation of cortisol to cortisone or vice versa depends on the cofactors available for the reaction (Stewart et al., 1988). Both 11β-HSD isoforms are membrane bound, hydrophobic enzymes that bind to either cortisol/cortisone or CORT/11-DHC, however the functions of these enzymes differ (Stewart et al., 1988).

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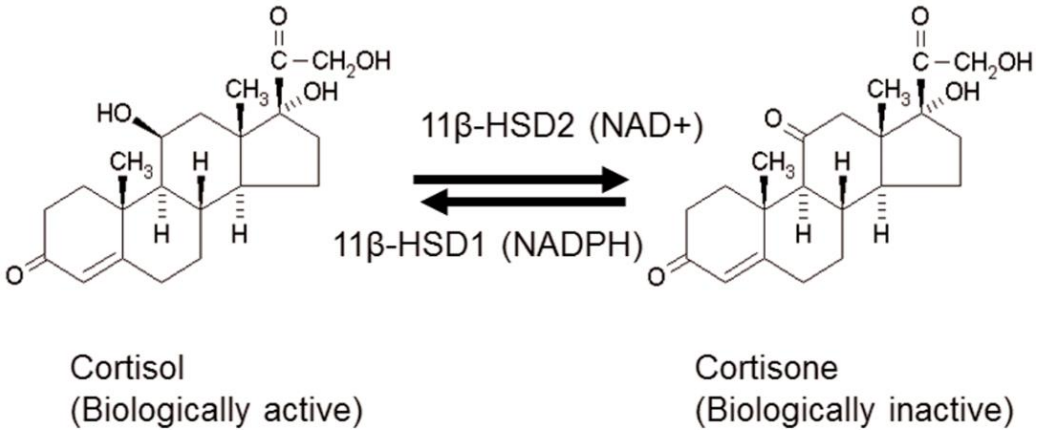

Fig. 3.16: 11β-HSD1 and 11β-HSD2 catalyze the interconversion of cortisol and cortisone. Reproduced from Hu et al., 2013.

In the presence of $NADP^+$, cortisol is oxidized to cortisone and in the presence of NADPH cortisone is reduced to cortisol by 11β-HSD1. However, the oxoreductase activity of 11β-HSD1 requires relatively high ratios of NADPH:NADP. These high ratios are maintained through the regeneration of NADPH from NADP during the conversion of glucose-6-phosphate to 6-phospho-gluconolactone which is catalyzed by hexose-6-phosphate dehydrogenase (Cooper and Stewart, 2009). The reduction of cortisone is, however, the dominant reaction in cells transfected with recombinant 11β-HSD1. Furthermore, the enzyme can only catalyze the reactions in the presence of relatively high concentrations of steroid (Moore et al., 1993). Synthetic glucocorticoids that are commonly used in *in vivo* experiments, such as prednisolone (an 11-ketosteroid), often lack biological activity, which can be induced by reducing these steroids to their 11β-hydroxylated states. This transformation is often catalyzed by 11β-HSD1 in the liver (Agarwal et al., 1990).

Although 11β-HSD1 and 11β-HSD2 do not display a high percentage of sequence identity, they perform physiologically related functions but catalyze opposing reactions. 11β-HSD2 is unidirectional and catalyzes the conversion of cortisol to cortisone through oxidation, utilizing NAD^+ and is expressed in mineralocorticoid target tissues to inactivate glucocorticoids and ensure that only mineralocorticoids elicit effects in these tissues (Oakley and Cidlowski, 2013; Rusvai and Naray-Fejes-Toths, 1993).

Corticosteroid transport

Once produced in the adrenal, the hormones are secreted into the bloodstream and transported to target tissues. Steroids are generally transported by three protein carriers — albumin, corticosteroid-binding globulin (CBG) and sex hormone binding globulin (SHBG) (Hammond,

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2016). The interaction between these transporters and the steroid hormones controls the free (active) vs bound (inactive) ratio of steroids in circulation and thus controls the capacity of the steroids to access their intended sites (Siiteri et al., 1982). It has generally been assumed that unbound steroids are the active steroids which would elicit downstream responses in target tissue with most disease states linked to steroid imbalances being diagnosed based on circulatory steroid levels (Mendel, 1989). However, this view may be naïve and an oversimplification as the ability of steroids to reach their site of action depends on compartmentalization, vascular permeability, extravascular fluid and extracellular matrix constituents, and the position of the target cells within the target tissues (Hammond, 2016).

Albumin is typically a low affinity, low specificity and high abundance carrier, whereas CBG and SHBG constitute high affinity, high specificity and low abundance plasma protein carriers. Albumin binds to the majority of steroids and although its affinity for the steroid substrate is relatively low (µM range), it is present in relatively high concentrations in plasma, approximately 1000-fold higher than CBG or SHBG, allowing it to buffer free versus bound steroid concentration effectively (Dunn et al., 1981). ALDO is mainly transported by albumin in the bloodstream, whereas DOC, which elicits mineralocorticoid-like effects, is transported by CBG.

CBG, from the serine proteinase inhibitor family, binds to free plasma glucocorticoids and PROG, which are cleaved from CBG by specific proteases at the site of delivery. CBG is synthesized in the liver, however, studies have shown that extra-hepatic biosynthesis may occur in the kidneys of immature rodents. Mice pups displaying CBG inefficiencies, do however not show any abnormal renal development (Scrocchi et al., 1993). Similarly, humans presenting with CBG inefficiencies may present as hypotensive patients, however no associations have been made with renal disease in these cases (Emptoz-Bonneton et al., 2000).

Initial studies suggested that the steroid binding sites of CBG were located in the core of the protein, however, it has been confirmed that these sites are located on the surface of the protein (Bolton et al., 2014; Gardill et al., 2012), showing that the steroids react with specific amino acid residues within the steroid-binding site (Lin et al., 2010). The studies showed the mechanism through which CBG bound steroids with high affinity, and that proteolysis of the reactive center loop (RCL) resulted in the irreversible loss of binding. Furthermore, it was shown that natural mutations within the essential residues of the binding site resulted in the loss of the binding affinity of CBG for glucocorticoids (Lin et al., 2010). Variants of CBG with

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differing cortisol binding abilities complicate the calculation of free cortisol, which is often used as a biomarker in diseased states (Bae, 2015; Henley et al., 2016).

Acute inflammation results in a rapid decrease in plasma CBG, associated with the proteolysis of the RCL, resulting in an increase in circulating albumin-bound and free cortisol levels. In addition, increased adrenal glucocorticoid output, as a result of adrenal stimulation by ACTH, may also exacerbate the reduced steroid binding capacity of CBG, further leading to increased free cortisol levels. Increased glucocorticoid and interleukin-6 (IL-6) production during inflammation also contributes to the down-regulation of CBG production in the liver and in so doing maintains low CBG plasma levels. In the recovery phase after the inflammatory response, the CBG levels are returned to normal (Smith and Hammond, 1992).

Glucocorticoid and mineralocorticoid receptors

Steroid receptors exert differential control of gene expression influencing development, reproduction and homeostatis in eukaryotes. Steroid receptors, also known as class I nuclear receptors, include the PROG receptor (PR), estrogen receptor (ER), androgen receptor (AR), GR and MR (Mangelsdorf et al., 1995). After biosynthesis and transport, corticosteroids elicit their biological effects through interactions with the GR and MR in target tissue. In this section, we discuss the structural domains of steroid receptors and the mechanism of action of the GR and MR.

Structural domains of steroid receptors

Steroid receptors are similar in structural organization as is shown in Fig 3.17 and early investigations using partially purified nuclear receptor proteins showed two distinct subunits — a ligand-binding domain (LBD) located at the C-terminal of the protein and a DNA-binding domain (DBD) located towards the center of the receptor (Birnbaumers et al., 1983). The LBD is moderately conserved and, as the name suggests, contains a highly specific interior binding site for its ligand as well as a transcriptional activation function (AF-2) which is regulated by the ligand. AF-2 is involved in the recruitment of co-activating proteins, which in turn, influence the general transcriptional activation components and is involved in activating chromatin-remodeling proteins (Xu and Li, 2003). The LBD is also involved in self-assembly reactions, such as dimerization and tetramerization, which are essential for DNA-response element binding (Kumar and Chambon, 1988).

The DBD anchors the receptor to elements within the receptor-regulated promoters, while also transmitting information allosterically from other receptor domains. The LBD and DBD are

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joined via the hinge region consisting of a short amino acid sequence. Although there is still some speculation regarding the functionalities of the hinge region, studies have shown that it increases transcriptional activation after undergoing phosphorylation (Lee et al., 2006). AF-1 is located to the N-terminal side of the DBD and functions as a transcriptional activator in a ligand independent manner. AF-1 can, however, also synergize with AF-2 (Takimoto et al., 2003).

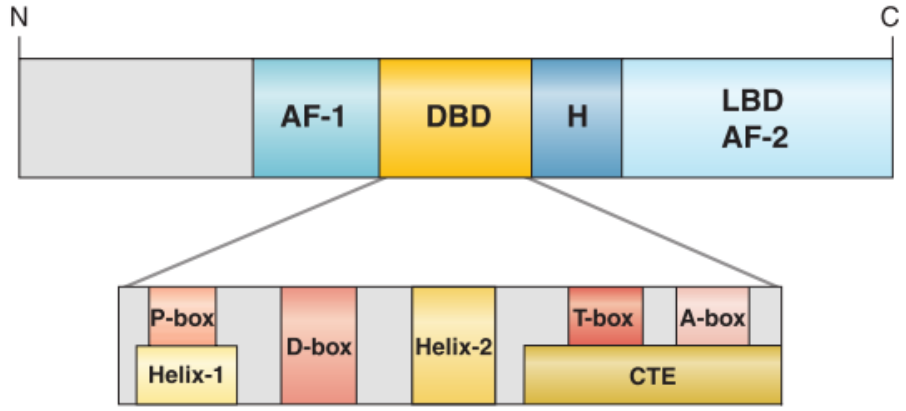

Fig. 3.17: Schematic representation of the structural organization of steroid receptors. AF, activation function; DBD, DNA-binding domain; H, hinge; LBD, ligand-binding domain. The components of the DBD is shown as an insert below the schematic of the receptor, illustrating the relative locations of the P-box, D-box, T-box, A-box, helix 1, helix 2, and the C-terminal extension (CTE). Reproduced from Bain et al., 2007.

Crystallographic investigations have shown that the LBD consist of 12 α-helices that form three antiparallel helix sheets, described as the α-helical sandwich. The receptor's ligand binding site is located in the inside of the structure and is lined by the helixes. The affinity and specificity of the LBD toward the ligand is determined by the hydrophobicity of the active pocket that is aligned to the shape of the ligand. In addition, several polar groups within the active pocket assist in binding and orientating the ligand (Bledsoe et al., 2002; Li et al., 2005). The AF-2 region within the LBD is involved in steroid coactivator recruitment and is found in a hydrophobic groove on the LBD, consisting of numerous helices including helix 12.

Agonist ligands are able to modulate the conformational mobility of the LBD and helix 12 and in so doing, regulate the interactions between the LBD and cofactors. The LBD has two possible conformations in the absence of an agonist: 1) helix 12 is positioned away from the core of the LBD resulting in an incomplete hydrophobic groove (Bourguet et al., 1995) or 2) a range of varying conformations within the domain with only a few in their active form (Johnson et al., 2000). Cofactor recruitment is induced after the binding of the ligand to the hydrophobic

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groove and the stabilization of helix 12. The latter can occur through ligand-stabilized helices close to the binding site (Shiau et al., 1998), direct interaction between the ligand and helix 12 (Bledsoe et al., 2002) or allosteric interactions between the coactivator-binding and ligand-binding pockets (Nettles et al., 2004). Antagonists block cofactor binding by allowing helix 12 to bind to the hydrophobic groove, mimicking cofactor binding and resulting in altered transcriptional activities (Shiau et al., 1998). Co-repressors may also alter transcriptional activities through sterically hindering helix 12 and preventing the formation of the hydrophobic groove (Xu et al., 2002). The presence of an antagonist ligand could further stabilize the binding of the co-repressor by creating a greater area for co-repressor binding.

The GR DBD, one of the first receptor subunit structures to be elucidated, is still used as a representative model for steroid receptors. The globular DBD consists of two zinc-finger structures, each consisting of four cysteine residues and a zinc atom (Freedman et al., 1988). Further structural components of the DBD include helix 1 (N-terminal helix) and helix 2 (C-terminal helix). These helices contribute to the overall stability of the DBD through interactions with the major groove. Binding of the GR to the DNA-binding via the DBD may induce dimerization (Rastinejad et al., 2013), and the dimer interface consists of residues that are found in the C-terminal zinc finger, called the D-box. The P-box, on the other hand, consists of residues that are paramount to sequence-specific DNA binding and is found in helix 1. Dimerization requires interactions between the DNA binding site and the P-box, which in turn induces conformational changes within the D-box and results in the recruitment of the second monomer (Baumann et al., 1993).

The sequences located on the N-terminal side of the DBD are mostly unstructured and do not display strong folding associations. However, secondary and tertiary folding can be induced through the interaction of molecules such as DNA or proteins or through altering the conditions of the solution. The effect elicited by DNA on the N-terminal structures of steroid receptors confirms that promoter binding sites act as functional ligands by influencing the receptor allosterically (Lefstin and Yamamoto, 1998).

Mechanisms of GR signalling

The GR, like the steroid receptors discussed above, consists of 3 domains — the transactivation domain (N-terminal with AF-1), the DBD which binds to the glucocorticoid response elements (GREs) and the LBD (C-terminal) (Fig 3.18) (Kumar and Thompson, 2005). In addition to the

structures described above, two nuclear localization signals (NL1 and NL2) have been identified and are found within the LBD and in the DBD/hinge region.

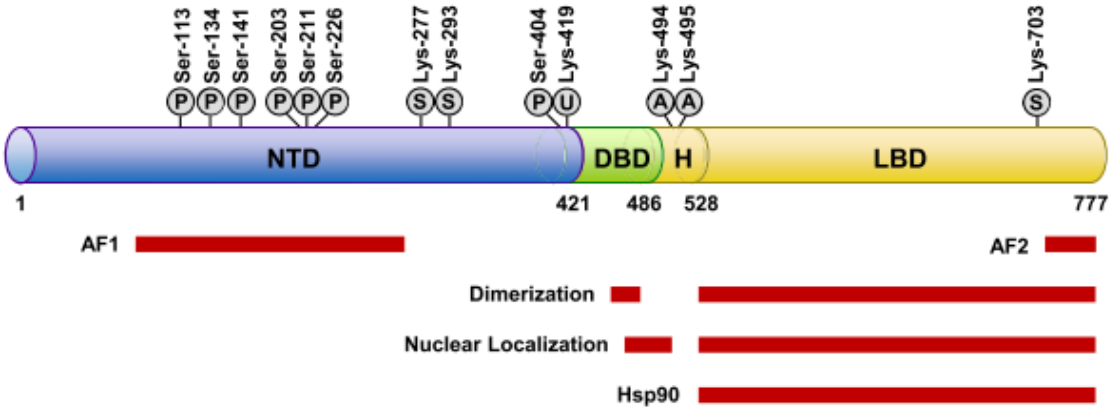

Fig. 3.18: Representation of the human GR domains. Potential sites for posttranslational modifications with amino acid residues that would be subject to modification are shown, as well as the AF-1 and 2 receptor domains and receptor regions involved in transactivation, dimerization, hsp90 binding and nuclear localization. Reproduced from Oakley and Cidlowski, 2013.

The GR is predominantly found in the cytoplasm in its unliganded form, where it is bound to chaperone proteins (mainly heat shock proteins) and immunophilins (Pratt and Toft, 1997). The association with these proteins is significant as the GR is maintained in a transcriptionally inactive form that favours ligand binding. After dissociating from CBG, free cortisol diffuses across the plasma membrane and, depending on the expression of 11β-HSD isoforms, is available for binding to the GR. After ligand binding, the GR undergoes a conformational change and the associated chaperone proteins dissociate from the GR-ligand complex. The conformational change exposes NL1 and NL2, which results in the GR-ligand complex translocating to the nucleus. The GR-ligand complex subsequently binds to the GRE in the nucleus and influences the expression of target genes (Beato, 1989) (Fig 3.19). The GRE, consisting of two 6-base pair half sites, is an imperfect palindrome (GGAACAnnnTGTTCT) and each half site is occupied by one homodimer GR subunit. The three nucleotides located between the two 6-base pair DNA binding sites, is essential for GR dimerization on the GRE. This GRE, often referred to as an activating or positive GRE, has always been associated with transactivation of gene transcription, although recent studies have shown that target gene transcription can also be repressed via a GRE (Uhlenhaut et al., 2013). The latter suggests that parameters outside of the GRE sequence could regulate whether a GRE is positive or negative (nGRE) (Surjit et al., 2011). The palindromic nGRE sequence CTCC(n)0-2GGAGA was found

to differ from that of the positive or classic GRE and does not result in the dimerization of two GR monomer subunits (Hudson et al., 2013). Although this motif was shown to elicit repressive effects and is abundant throughout the human genome, the extent to which this motif is utilized by the GR for repression requires further investigation. In addition, a recent study found that helix 8 is critical for ligand binding and receptor function (Deng et al., 2015).

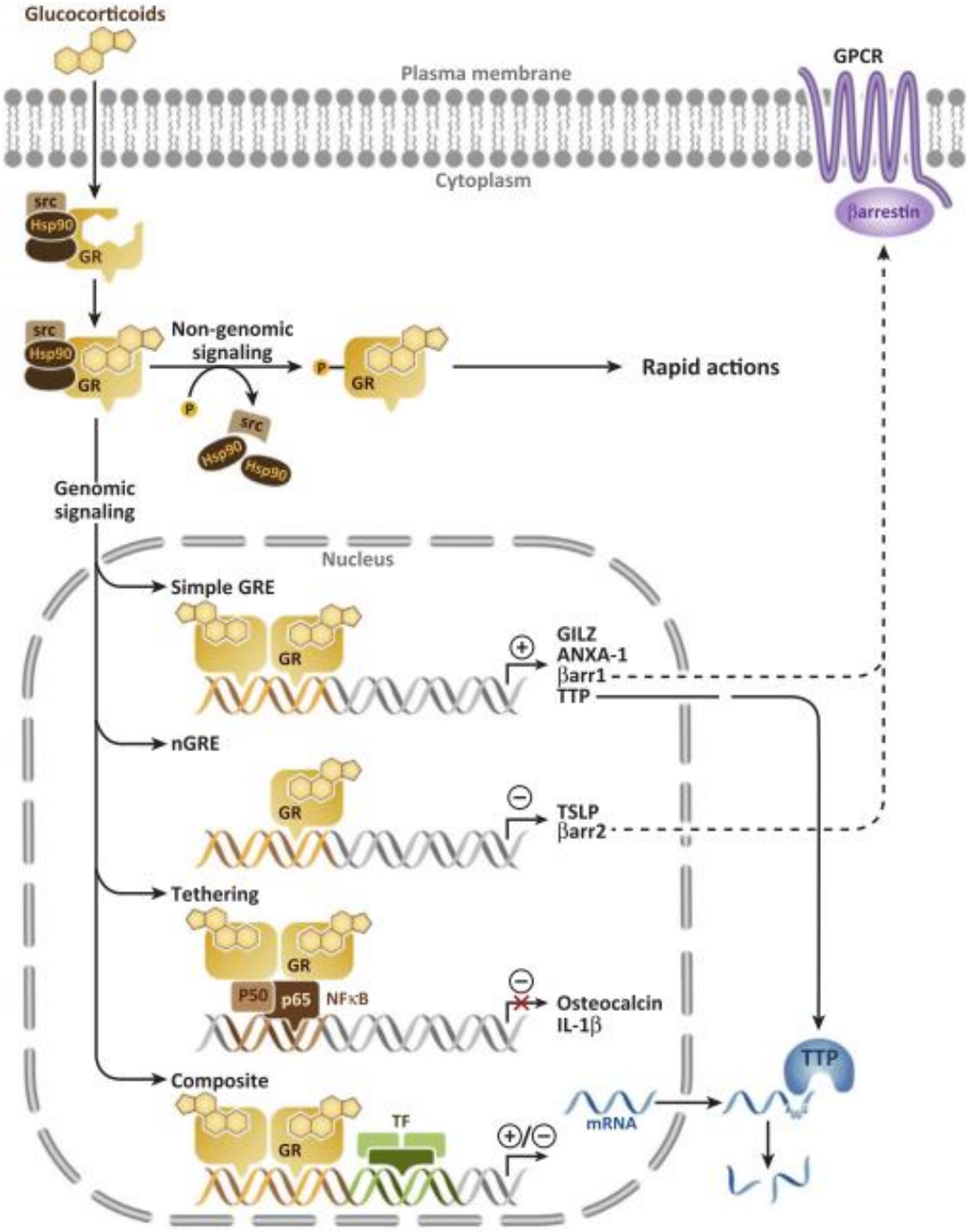

Fig. 3.19. GR signalling mechanisms. Glucocorticoids bind to the cytoplasmic GR which induces a conformational change in the GR, leading to its hyperphosphorylation (P) and subsequent dissociation from accessory proteins, - translocation into the nucleus. In the nucleus, GR-signalling occurs through simple, tethering and composite mechanisms, whereas

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the non-genomic effects of glucocorticoids are mediated through cytoplasmic or membrane bound GR. Reproduced from Kadmiel and Cidlowski, 2013.

Typically two mechanisms of GR signalling exist — non-genomic and genomic (which includes simple, tethering and composite mechanisms) (Fig 3.19). Simple GREs, as discussed above, consist of imperfect palindromes with two 6-base pair DNA binding sites which are separated by three nucleotides. Sixty percent of these DNA sequences within simple GREs are highly variable and thus determine the GR mediated transcriptional activity (Meijsing et al., 2009). Liganded GR binding to simple GREs is usually associated with transactivation, which involves the recruitment of coactivators (Lonard and O'Malley, 2007). Composite GREs, consisting of binding sites that accommodate the binding of the GR and other transcription factors, allow the GR to modulate gene transcription in a similar manner as above. The tethering mechanism, however, has been mostly associated with transrepression of gene transcription. The suppression of inflammatory gene transcription in diseases such as asthma or chronic obstructive pulmonary disease (COPD), relies on the tethering of the GR to transcription factors such as activator protein-1 (AP-1), NF-kB and signal transducer and activator of transcription 3 (STAT3) (Kassel and Herrlich, 2007).

In addition to GR binding occurring in a tissue specific manner as a result of chromatin accessibility and sufficient accessibility of the GRE, evidence suggests that only a small percentage of GREs are occupied by the GR (John et al., 2011). Although it is known that the tissue-specific effects of glucocorticoids are influenced by GR levels and the differential expression of cofactors (Oakley and Cidlowski, 2013), it has been proposed that the tissue-specific effects of glucocorticoids may be due to the type of GRE available for binding by the GR (Reddy et al., 2012). This may offer an explanation for the hypersensitivity of some tissues towards glucocorticoids and the difference in glucocorticoid concentrations required to elicit their effects.

The non-genomic effects of glucocorticoids occur within minutes and may affect membrane fluidity, interactions with cytoplasmic and membrane proteins or lipids and neurotransmission (Haller et al., 2008). Although these effects can be mediated through glucocorticoids binding to cytoplasmic or membrane GRs, glucocorticoids may also exert non-genomic effects by binding directly to other receptors such as $GABA_A$ receptors (Strömberg et al., 2005).

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Selective Glucocorticoid Receptor Agonists and Modulators (SEGRAMs)

For many years the dimer-mediated transactivation of gene transcription by the GR was associated with the side effects associated with glucocorticoid treatments, whereas the monomer-mediated transrepression of gene transcription was associated with the beneficial side effect profile. In theory, this concept allowed for a distinction between the two transcriptional processes and a potential drug target for the treatment of glucocorticoid excess disorders that elicits an anti-inflammatory effect without the side effects (although not all pro-inflammatory effects are deemed unfavourable and not all anti-inflammatory effects are deemed favourable). This concept led to the discovery of selective GR agonists (SEGRAs) or modulators (SEGRAMs), previously known as dissociated glucocorticoids, that could cause GR mediated transrepression of transcription, without transactivation of gene transcription. Compound A is a well-known example of a SEGRA, and is known to mediate transcriptional repression via the GR, but display no transactivation activity (de Bosscher et al., 2008, 2005). The study concluded that the effects of Compound A would be anti-inflammatory with no side effects.

However, the recent discovery that transactivation via the GR can also lead to anti-inflammatory effects through binding to a nGRE, together with the fact that GR activity may be selectively modulated by a ligand inducing a specific GR conformation (Vandevyver et al., 2013), has led to the reassessment of the SEGRAM concept. The concepts of selective monomerizing GR agonists and modulators (SEMOGRAM), eliciting effects via GR monomers, and selective dimerizing glucocorticoid receptor agonists or modulator (SEDIGRAMs), eliciting effect via GR dimers, was subsequently proposed (de Bosscher et al., 2016). Thus, the current evidence in the literature suggests the pro- and anti-inflammatory effects elicited via the transcriptional activities of the GR may be due to different ratios of GR-complex conformations, i.e. monomers versus dimers, rather than the classical transactivation versus transrepression approach (de Bosscher et al., 2016; Sundahl et al., 2015).

Mechanisms of MR signalling

The MR has been shown to bind to various steroids with high affinity, most notably to ALDO, DOC, CORT (in rodents), cortisol (in humans) and PROG (Sutanto and de Kloet, 1991). ALDO is considered to be the primary MR ligand (Bledsoe et al., 2005), although studies have shown that cortisol acts as the primary MR ligand (agonist) in some target tissues, while PROG is mostly associated with antagonistic effects (Fagart et al., 1998).

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The MR is structurally similar to the GR and Fig. 3.20 shows the basic domains and activation factors involved in the regulation of gene transcription by mineralocorticoids via the MR. The LBD and N-terminal domains are of great importance with regard to regulation and the MR LBD has been found to be involved in binding of ligands, transport to the nucleus, interactions with heat shock proteins and transactivation of target gene expression through cofactors and ligand dependent mechanisms (Farman and Rafestin-Oblin, 2001; Pippal and Fuller, 2008). ALDO binds to a fully enclosed pocket of the LBD, which causes the repositioning of helix 12 to form the hydrophobic cleft which allows for cofactor binding and subsequent activation of MR mediated gene transcription (Bledsoe et al., 2005). AF-2 comprises of not only helix 12, but requires other areas of the LBD to part take in the activation function. In the N-terminal domain, AF-1 plays a significant role in activating gene transcription. AF-1, other than AF-2, is not ligand dependent and has been localized to amino acid residues 328 – 382 (Fuse et al., 2000). Studies have suggested that AF-1 comprises two distinct activation factors, although not all results are in agreement (Govindan and Warriar, 1998).

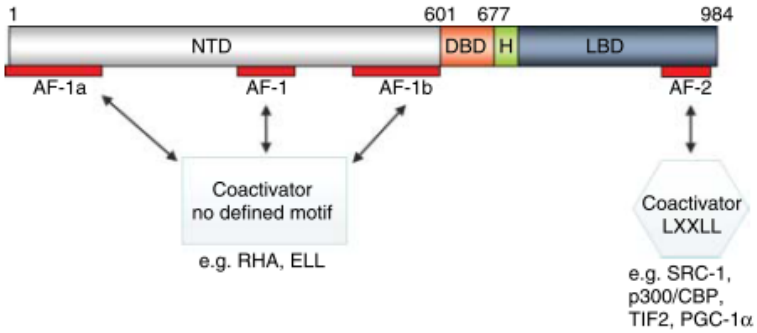

Fig. 3.20: Representations of structural domains of the human MR and cofactor interactions. Reproduced from Yang and Young, 2009.

The unliganded MR is found in the cytoplasm and, similar to the GR, is maintained in an inactive form bound to chaperone proteins (Gomez-Sanchez et al., 2006,) which dissociate when the MR undergoes a conformational change due to ligand binding to the MR. The ligand bound MR, mono- or heterodimer, translocates to the nucleus where it binds to a specific DNA target, called a mineralocorticoid response element (MRE), influencing the transcription of target genes (Hellal-Levy et al., 2000) (Fig. 3.21). Rapid, non-genomic effects have also been associated with MR signalling and the most renowned of these are the activation of protein kinase C, activation of Na^+/H^+ exchange and elevated intracellular calcium (Funder, 2005).

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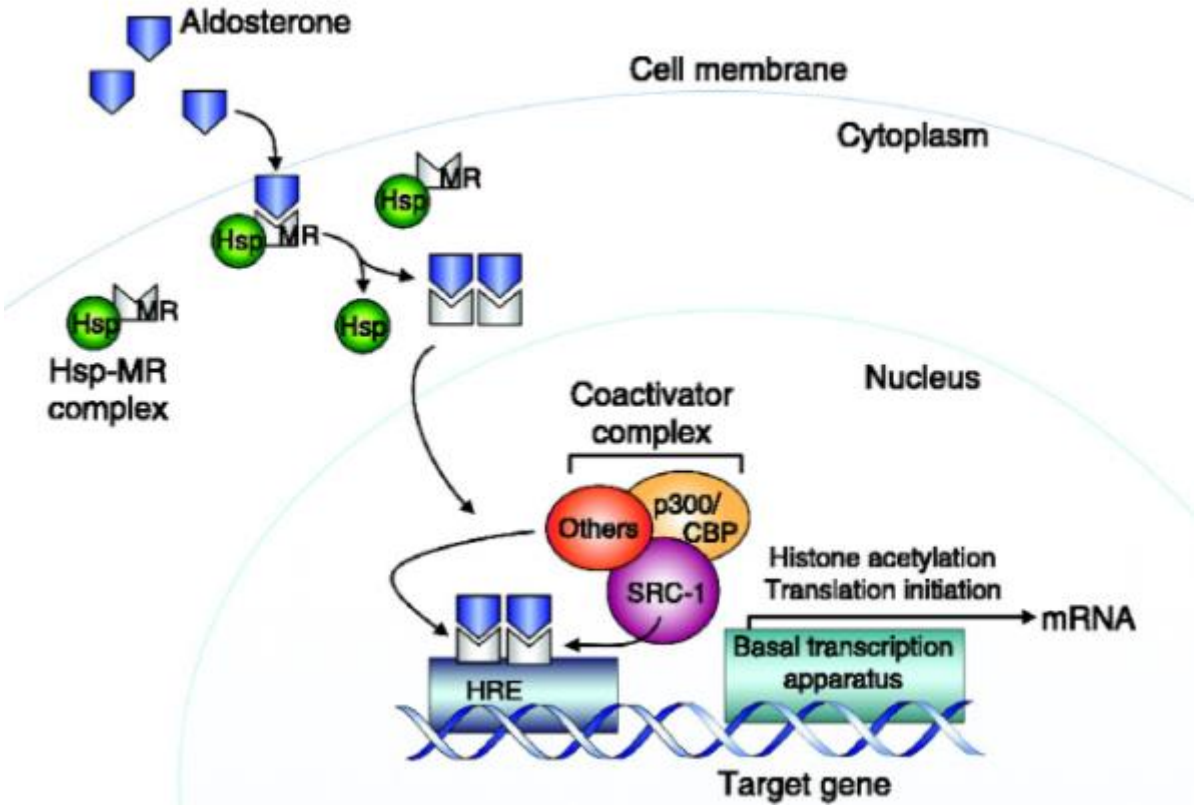

Fig. 3.21: Transactivation mechanism via the MR. The heat shock proteins dissociate from the liganded MR in the cytoplasm and nuclear translocation is induced. In the nucleus the MR-ligand complex binds to the hormone response element (HRE) where after cofactors, such as SRC-1 and p300/CBP, are recruited to the MR to accommodate the acetylation of histones and for gene transcription to take place. Reproduced from Yang and Young, 2009.

Gene transcription via the MR can be activated by cortisol and ALDO binding as the MR has equal affinity for these ligands (Arriza et al., 1987), however the differential activation of the MR by these ligands is associated with varying effects at different target sites. The differential activation via the MR may take place on three levels — pre-receptor, receptor and post-receptor. The inactivation of cortisol by 11β-HSD2 represents the pre-receptor regulatory mechanism. As cortisone cannot bind to the MR, no activation of gene transcription via the MR would occur (Funder et al., 1988). It is thus unlikely that the MR mediated gene transcription will be activated by either cortisol or cortisone and will occur though the binding of ALDO to the MR. The pre-receptor intervention is only applicable in epithelial cells in the kidney, blood vessel walls, colon and sweat glands as 11β-HSD2 is expressed minimally in other tissues (Galigniana et al., 2004). At receptor level, the differential activity of the MR is linked to the conformational changes induced by the binding of either cortisol or ALDO. ALDO induces greater transcriptional activation than cortisol and dissociates at a much slower rate (at any cortisol concentration) (Li et al., 2005). Furthermore, the stabilization of ligand

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binding to the MR through exchanges between the MR N-terminal and hinge region on the LBD are much greater in the presence of ALDO, suggesting further ligand specificity by the MR (Li et al., 2005).

Post-receptor effects that contribute to MR ligand selectivity refers to the transcription of different MR isoforms that differ in their ligand specificity which may further contribute to MR ligand selectivity. Human MR transcription can be regulated by two promotors (P1 and P2) that result in the hMRα and hMRβ transcripts, respectively. These isoforms differ in their 5' untranslated regions and are expressed in different tissues. Transcription by P1 can be induced by the presence of glucocorticoids, however P2 is sensitive to induction by mineralocorticoids, which adds to MR specificity (Zennaro et al., 1997).

Physiological effects of corticosteroids

The physiological effects elicited by corticosteroids depend on many factors as described above. The main effect of these hormones is to maintain a homeostatic state in the body, however for the purposes of this study we will focus on the psychiatric and cognitive, immunomodulatory and cardiovascular effects.

Psychiatric and cognitive effects

Psychiatric side effects that are associated with corticosteroid treatment have been reported and appear to be dose dependent — the higher the dose, the greater the probability of developing psychiatric complications (Goldstein and Preskorn, 1989). These side effects have been observed in spite of the different methods of administration (Berthelot et al., 2013). Although it has been shown that women and young children are more prone to develop psychiatric related side effects when receiving corticosteroid treatment (George et al., 2004), a direct link between age and gender has not been established. Although an exact pattern of the onset of psychiatric side effects after the initiation of corticosteroid treatment has not been established, research suggest that it is most probable that side effects start within the first five days of treatment (Drozdowicz and Bostwick, 2014; Ling et al., 1981).

The hydrophobic nature of corticosteroids allows them to permeate across the plasma membrane, as well as the blood brain barrier, and influence gene transcription by binding to the GR and/or MR. In addition to their endocrinological effects, corticosteroids affect the feeling of hunger, the processes of learning and memory, as well as the sleep-wake cycle. Behavioral disorders resulting from corticosteroid treatment includes restlessness, weight gain associated with increased appetite (70 % of cases) and insomnia (73 % of cases) (Da Silva et

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al., 2006). Prolonged corticosteroid exposure has also been associated with a sense of well-being labelled “steroid euphoria” as well as muscle stiffness and catatonia related behaviours (Benyamin et al., 2008).

These effects are mediated through interactions with specific receptors located in the basolateral amygdala, hippocampus and prefrontal cortex (Lewis and Smith, 1983). Glucocorticoids in the brain regulate their effects through neurotransmission with particular focus on serotonin and dopamine neurotransmission. Previous studies have observed a strong link between high endogenous cortisol concentrations and hippocampal atrophy associated with cognitive dysfunction (Fardet et al., 2012; Hall et al., 1978). In a healthy subject, the damage caused by high cortisol concentrations would be inhibited by the feedback inhibition of the HPA-axis and the resulting decrease in plasma steroid concentrations.

The susceptibility of individuals to stress-related psychopathology appears to vary considerably and has been linked to variations in the sensitivities and functionalities of the HPA-axis, which is greatly determined by GR sensitivity (Harris et al., 2013). The latter may impact on the effect of a stressor on the brain, HPA-axis feedback actions and the ultimate development of depression. The transcription of GR mediated genes are additionally regulated by factors such as FK506 binding protein (FKBP5), which activates a short negative feedback loop after binding to the liganded GR and decreases GR signalling (Binder, 2009). The MR, however, regulates the initiation of the stress response through transcriptional activities of genes associated with HPA-axis activation. The functional role of MRs in the maintenance of basal steroid levels has been suggested to occur through inhibitory actions mediated through GABAergic neurons that act on the PVN of the hypothalamus (Berardelli et al., 2013). In addition, the MR contributes to the exertion of rapid non-genomic effects though neurotransmission and the synaptic signalling strength in the brain (de Kloet et al., 2016, 2005; Hauer et al., 2011).

In addition to the effect elicited by the conventional genomic signalling pathways, GR and MR have been shown to adapt to stressors within minutes. These rapid effects have been associated with non-genomic effects of the GR and MR, and it has been shown that membrane bound GR and MR exists within the brain tissues and are responsible for eliciting rapid effects through signalling pathways that mirror the conventional signalling strategies (Groeneweg et al., 2012).

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Immunomodulating effects

In spite of the well-documented side effects of glucocorticoids, they remain the gold standard for the treatment of inflammatory disorders and immune suppression after organ transplants. Beneficial and adverse effects are elicited through the ability of glucocorticoids to act on nearly every cell type within the immune system (Zen et al., 2011). In dendritic cells, cell maturation is suppressed by glucocorticoids resulting in cells with weak T-cell stimulating activities. In addition to inducing apoptosis and migration of dendritic cells, glucocorticoids also elicit varied effects depending on the GR isoform expressed in these cells (Cao et al., 2013). Glucocorticoids have also been shown to increase the pro-inflammatory response initiated by macrophages. Typically, macrophages contain Toll-like receptors that detect harmful stimuli, such as infectious agents, resulting in the activation of the pro-inflammatory response through the inflammasome complex (Guo et al., 2015). Also, Toll-like receptor-2 is activated by a glucocorticoid-TNF-α complex which results in the stimulation of innate immunity (Hermoso et al., 2004). Contrary to the pro-inflammatory effects, glucocorticoids have been shown to suppress the inflammatory response by augmenting the phagocytosis of macrophages as well as neutrophils (Baschant and Tuckermann, 2010). Neutrophil migration into the inflammatory site is also suppressed by glucocorticoids by inhibiting adhesion molecule expression (Busillo and Cidlowski, 2013). The effects of glucocorticoids on T-cells may vary considerably and depend on the type of T-cell. Glucocorticoids induce apoptosis in pro-inflammatory T-cells, whereas the survival of regulatory T-cells is augmented by the actions of glucocorticoids (Baschant and Tuckermann, 2010). In auto immune disorders and B-cell tumors, glucocorticoids inhibit the production of B-cell antibodies with additional inhibitory effects on the activation, proliferation and differentiation of B-cells (Zen et al., 2011).

The anti-inflammatory and anti-proliferative effects of glucocorticoids render them popular treatment choices for cutaneous inflammatory disorders such as psoriasis or eczema (Goldminz et al., 2012), although the beneficial effects may be accompanied by skin atrophy and delayed wound healing. The identification of skin specific GRs showed that the anti-proliferative effects of glucocorticoid treatments are associated with transrepression via the GR in keratinocytes, with delayed wound healing being associated with both transactivation and transrepression (Sevilla et al., 2013). Cutaneous therapies could thus be enhanced by utilizing treatments that augment transcriptional activities linked to transrepression via the GR instead of transactivation.

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Cardiovascular effects

Corticosteroids have been linked to the initiation and progression of cardiovascular diseases with hypertension and heart failure being the most common clinical conditions. One of the first studies suggesting that ALDO may play a significant role in heart failure showed that increased plasma ALDO levels resulted in a predictive index of morbidity and mortality (Swedberg et al., 1990). In a more recent investigation it was found that both plasma ALDO and cortisol are complementary predictors of mortality (Güder et al., 2007). Further investigations showed that ALDO was able to induce cardiac fibrosis without affecting blood pressure significantly, which was prevented following treatment with spironolactone (SPL), a well-known MR antagonist (Robert et al., 1994; Young et al., 1994). The latter discovery prompted investigations into MR antagonists in the treatment of cardiovascular disease. The increased interest in the MR as drug target led to the discovery that the MR is expressed not only in the well-known ALDO target tissues, but in the blood vessels (Meyer and Nichols, 1981), heart (Funder et al., 1988; Lombès et al., 1992) and brain (Yu et al., 2002). During chronic heart failure, the elevated concentrations of AngII, a potent stimulator of ALDO biosynthesis, and ALDO may have damaging effects on cardiovascular structure and although ACE inhibitors may decrease the plasma concentrations of ALDO and AngII (Pitt, 1995), this effect is dissipated after a few months of treatment. The mechanism though which ALDO and AngII elicit the latter "escape" effect is still largely unknown.

The ligand responsible for the activation of the MR in cardiomyocytes has been a point of debate in recent years as it is known that the MR binds to ALDO, cortisol, CORT and DOC with high affinity. In the distal colon, distal nephron, sweat glands and other MR classical target tissues, the MR is co-expressed with 11β-HSD2 that encourages ligand specificity of the MR as this enzyme converts cortisol to cortisone. In vascular smooth muscle cells and cardiomyocytes, very little or no 11β-HSD2 activity has been reported, suggesting that glucocorticoids, which are present approximately 1000-fold more than ALDO (Rickard et al., 2009), can interact with the MR.

In addition to the effects of the glucocorticoids, ALDO acts as an agonist for MR mediated gene transcription in cardiomyocytes and vascular smooth muscle cells. ALDO was shown to increase Ca^{2+} channel activity through the redistribution of Ca^{2+} ions across the membrane in the cardiomyocytes (Gomez et al., 2009; Ouvrard-Pascaud et al., 2005). The beating frequency of cardiomyocytes was significantly affected by the presence of ALDO, however the treatment of the cardiomyocytes with CORT or dexamethasone (Dex), a potent synthetic glucocorticoid,

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in an environment favouring MR-glucocorticoid binding, had no significant effect on the increased beating response elicited by ALDO (Rossier et al., 2008). In addition, ALDO and CORT resulted in a similar size in myocardial infarction and in both cases the effects were ablated by the addition of SPL (Latouche et al., 2010; Mihailidou et al., 2009).

Although the MR displays similar affinities for corticosteroid ligands, it has been reported that the activation efficiencies of the MR:ALDO and MR:cortisol complexes differ, with the MR:ALDO being more stable and more efficient in transactivating gene transcription compared to the MR:cortisol complex. Hetero- and homodimerization of MR and GR isoforms that bind to the same HREs, expressed in a tissue specific manner, further add to the cell specific transcriptional activities of these receptors. Cell specific expression of cofactors, as well as phosphorylation sites in the LBD of the MR that influence ligand binding (Shibata et al., 2013), further add to the tissue specific transcriptional activities of these receptors (Gomez-Sanchez and Gomez-Sanchez, 2012; Savory et al., 2001; Tsugita et al., 2009). The N/C interaction, an interaction between the N-terminus and the LBD first identified for the AR (Li et al., 2006), has been identified as an important interaction in terms of MR activity and is antagonized by SPL and eplerenone (Pippal and Fuller, 2008). In terms of MR agonists, cortisol and DOC, unlike ALDO, do not mediate the N/C interaction and antagonized the N/C interaction mediated by ALDO. The latter adds to the discrimination between ALDO and cortisol binding to the MR and confirms that different ligands induce different conformational states (Fuller, 2015).

Studies have investigated the role of the MR in the progression of heart failure using MR antagonists, such as SPL and eplerenone (Grossmann and Gekle, 2009; Kimura et al., 2011). Although MR antagonists have been shown to be efficient treatments for heart failure and hypertension in clinical trials, the use of these antagonists have not been implemented to the extent that would be expected (Albert et al., 2009). SPL, marketed since the 1960's as an anti-hypertensive and diuretic drug, exerts useful clinical effects through the antagonism of the effects of ALDO on the MR (Piotrowski, 2012). However, this drug has been associated with painful menstrual disturbances in premenopausal women (Hughes and Cunliffe, 1988) which has been accredited to androgen-like and PROG-like effects. These side effects resulted in limited investigations into SPL as an anti-hypertensive treatment, however, the fairly recent increase in heart failure has contributed to renewed interest in SPL and other steroidal MR antagonists such as eplerenone (Piotrowski, 2012). It was however shown that SPL also exerts anti-glucocorticoid effects through interactions with the GR and androgenic effects through

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interactions with the AR (de Gasparo et al., 1987). The affinity of these receptors for SPL is however lower than that of the MR and thus the associated effects via the GR and AR are often negligible when compared to effects via the MR (Couette et al., 1992). Generally speaking, eplerenone has been shown to elicit fewer endocrine disrupting effects than SPL (Stier, 2003). In addition to steroidal MR antagonists, several nonsteroidal MR antagonists, such as finerenone (Liu et al., 2015), have been identified displaying reduced side effect profiles (Piotrowski, 2012).

Conclusion

Taken together, this chapter outlines the complex mechanisms that are involved in maintaining homeostasis. The influence of hormones, cytokines, neurotransmitters, receptors and delivery systems all play crucial roles in maintaining homeostasis during every-day or stress-like perturbations. The complexity of these processes and mechanisms further lends itself to being susceptible to disturbances between processes in such a way that if a disease state would affect one system, other systems would inevitably be affected, with a cascade of potentially detrimental effects. Chronic exposure to corticosteroids has been shown to elicit such responses as is evident through their psychological, immunomodulatory and cardiovascular effects as discussed in this chapter.

The studies discussed in this chapter also suggest several mechanisms through which corticosteroid actions can be manipulated, whether as a therapy or as a result of a disease state. The inhibition of steroidogenesis, the modulation of the HPA-axis and the transcriptional activities of the GR and the MR in target tissues may all be valid targets for the development of anti-depressive, anti-inflammatory and/or anti-hypertensive treatments. For example, the role of the HPA-axis, glucocorticoids and GR signalling in the inflammatory response and immunomodulation is well established and as such these three aspects of the inflammatory response have been identified as drug targets for the treatment of clinical conditions related to immune dysfunction (Edwards, 2012; Kadmiel and Cidlowski, 2013). In addition, the observation that hypersensitive GREs exist may suggest that lowered concentrations of glucocorticoids could be employed as therapies to treat specific disorders, avoiding the typical side effects associated with high concentrations of free glucocorticoids. Furthermore, targeting protective functions such as the co-expression of the MR and 11β-HSD2 in mineralocorticoid sensitive tissues, as well as cofactor recruitment, might also be a valuable target for drug development for diseases linked to excessive corticosteroids.

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Although these processes and mechanisms are valuable targets for the pharmaceutical industry for the treatment of corticosteroid-related diseases, it has been suggested that some botanicals exert their medicinal properties by influencing these processes and mechanisms. Extracts made from *Aspalathus linearis* (Schloms et al., 2012) and *Sceletium tortuosum* (Swart and Smith, 2015) have been shown to influence steroid output in adrenal cell models, with *Aspalathus linearis* also inhibiting individual steroidogenic enzymes. *S. frutescens* has been shown to elicit anti-anxiety effects in stressed rats by lowering serum CORT and influencing the expression of the $GABA_A$ receptor and the GR in the hippocampus (Smith and Myburgh, 2004b; Smith and van Vuuren, 2014). Specific compounds, that have been isolated from plant extracts, like triterpenoids, have also shown anti-anxiety and anti-inflammatory effects. Furthermore, various plant extracts have been shown to inhibit the catalytic activities of hepatic P450 enzymes.

In the next chapter we thus investigated the effect of a methanolic *S. frutescens* extract, as well as compounds isolated from the extract, on the catalytic activity of steroidogenic enzymes, adrenal steroidogenesis and the transcriptional activities of the extract and SUB via the MR and GR.

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Chapter 4: The influence of *S. frutescens* on adrenal steroidogenesis and downstream receptor-ligand interaction

Introduction

S. frutescens is generally used as a herbal remedy to treat disorders that are associated with inflammation and stress (van Wyk and Wink, 2004). The application of this herbal remedy as anti-stress and anti-inflammatory tonic may be rooted in the effect of *S. frutescens* on steroid hormone production, specifically cortisol biosynthesis.

Inflammation is initially a favourable defensive response to a stressor, which results in the production of pro-inflammatory agents with the objective to repair cellular damage or eliminate effects of an extracellular stressor. However, the re-establishment of homeostasis after a pro-inflammatory response is of utmost importance in avoiding chronic inflammation (Pascual and Glass, 2006). Inflammation is managed by the stress-induced release of glucocorticoids which, in addition to the stress response, play important regulatory roles in lipid, carbohydrate and protein metabolism, reproductive and growth processes and brain functions such as behaviour and memory (Chrousos and Kino, 2009). The physiological stress response is initiated when the brain detects a homeostatic challenge, be it physical, physiological or emotional, and results in the activation of the SNS. The release of epinephrine and NE by the SNS is followed by the activation of the HPA-axis. Stressors stimulate the secretion of ACTH from the pituitary within the HPA-axis to increase glucocorticoid production in adrenal steroidogenesis, resulting in increased active glucocorticoids in circulation. Chronic stress could thus result in the dysfunction of the HPA response and the disrupted adrenal steroidogenic output, resulting in glucocorticoid resistance and an inability to down regulate the inflammatory response (Cohen et al., 2012; Kanczkowski et al., 2013).

Previous studies have shown that a methanolic extract of *S. frutescens* significantly inhibited DOC (59 %) and PROG (17 %) binding in adrenal P450-containing mitochondrial and microsomal preparations. It was shown that the extract did not inhibit PREG binding to microsomal enzymes, however, a chloroform extract significantly inhibited PREG binding while an aqueous extract inhibited the conversion of PREG and PROG by ovine CYP17A1 (Prevoo et al., 2008, 2004). Furthermore, we have shown that deoxycortisol and DOC binding to P450 enzymes in adrenal mitochondrial preparations is inhibited in the presence of the methanol extract as well as cortisol and CORT production by CYP11B1, expressed in COS-1 cells (Sergeant, 2009).

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The therapeutic effects of *S. frutescens* have been attributed to the presence of a wide variety of compounds. The most renowned compounds identified in the extract are GABA, pinitol, L-canavanine together with flavonoid and triterpenoid compounds. The triterpenoids or SU compounds in *S. frutescens* extracts include four complex cycloartane glycosides –SU A, B, C and D with SUB being the major triterpenoid present in plant material (Fu et al., 2008), confirmed by liquid chromatography-mass spectrometry (LC-MS) (Albrecht et al., 2012; Avula et al., 2010). Recently, additional cycloartane glucosides were identified using novel chromatographic techniques (Zaki et al., 2016), however, limited information regarding these compounds is available.

Triterpenoid compounds in plant extracts have been linked to a variety of pharmacological effects which include lowering serum cholesterol levels, anti-inflammatory, cardiovascular and hypoglycaemic effects (Desai et al., 2009; Lockyer et al., 2012; Wu et al., 2011). The mechanisms by which these compounds elicit biological effects are mostly unknown, however, it has been suggested that these compounds are able to elicit steroid-like effects due to structural similarities with endogenous steroid ligands. Studies have shown that triterpenoids exhibit anti-cancer and anti-inflammatory properties with data indicating that many of these compounds target and downregulate NF-κB. Triterpenoids are furthermore widely used in Asian medicine in the treatment of chronic diseases which include amongst others, diabetes, obesity, cardiovascular atherosclerosis, arthritis and depression (Sanna et al., 2015; Venkatesha et al., 2016; Yadav et al., 2010). The aforementioned conditions are closely associated with an impaired endocrine system and, as such, triterpenoids may also interact with the steroidogenic P450 enzymes which catalyze steroid biosynthesis due to the structures of the compounds being closely related to the cyclopentanoperhydrophenanthrene structure of steroid hormones. Studies investigating the effect of *S. frutescens* extracts on hepatic P450 enzymes showed nevirapine metabolism by CYP3A4 to be inhibited in rats (Minocha et al., 2011), while the catalytic activity of CYP2D6 and CYP2E1 were not affected (Fasinu et al., 2013). As mentioned above, we have reported that *S. frutescens* extracts inhibit substrate conversion by steroidogenic P450 enzymes such as CYP17A1 and CYP21A2. It was suggested that the inhibition of P450 enzymes by the extract may be elicited by compounds within the extract that are structurally similar to natural steroid substrates (Prevoo et al. 2008; Prevoo et al. 2004). Besides interfering with the catalytic activity of the P450 enzymes, the triterpenoids may also mimic or antagonize the downstream actions of endogenous steroid hormones.

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We hypothesized that the anecdotal anti-inflammatory effects of *S. frutescens*, when used as a treatment in inflammatory disorders, could be attributed to the influence of the extract on steroidogenesis and downstream, on the activation of steroid receptors. We further hypothesized that the presence of triterpenoids, specifically SUB, in *S. frutescens* extract may contribute to its anecdotal effects. We therefore investigated the following:

- The effect of a methanolic extract of *S. frutescens* on single adrenal steroidogenic enzymes and steroid production.
- SUB levels in the extract used in this study.
- The effects of SUB on single adrenal steroidogenic enzymes and steroid production.
- The effect of the methanolic *S. frutescens* extract and SUB on downstream transcriptional activities elicited by the GR and MR.

Materials and Methods

Reagents

Phorbol 12-myristate 13-acetate (PMA), (9-α-fluoro-16-α-methylprednisilone (dexamethasone (Dex)), SPL, gamma aminobutyric acid (GABA) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (RSA). Steroids were purchased from Steraloids (Wilton, USA). COS-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's phosphate buffered saline, together with penicillin-streptomycin and trypsin-ethylenediaminetetraacetic acid (EDTA) were sourced from Gibco-BRL (Gaithersburg, MD, USA). *Mirus TransIT*®- LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Fetal calf serum was supplied by Highveld Biological (Lyndhurst, RSA), while cosmic calf serum was purchased from HyClone®,Thermo Scientific (RSA). *S. frutescens* subspecies (L.) R. Br. were supplied by Professor Ben-Erik van Wyk, Department of Botany of Rand Afrikaans University (Voucher specimen from Mrs. Grobler: C. Albrecht s.n sub B.-E. van Wyk 4126 (JRAU)). Triterpenoids were supplied by Prof J. Syce (University of Western Cape, Cape Town, South Africa) and Prof WR Folk (University of Missouri, Missouri, USA). All chemicals used were of high analytical grade and obtained from reputable suppliers.

S. frutescens extraction and isolation of SUB

S. frutescens was subjected to organic extraction using a glass soxhlet extractor coupled to a double wall condenser. Dried plant material, 18.5 g, was extracted with chloroform, 250 mL, for 8 hours, after which the solvent was replaced with methanol, 250 mL, and extracted for a

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further 8 hours (Swart et al., 1993). The methanol extract was dried at room temperature at reduced pressure on a rotary evaporator. The residue was redissolved in 35 mL de-ionized water and centrifuged at 6 000 × g for 5 minutes. The supernatant, with a final concentration of 53.43 mg/mL, was stored at -18 °C in aliquots until use. LC–MS/MS analysis of the extract was conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Milford, MA, USA) using a Waters BEH C18 column (2.1 x 50 mm, 1.7 µm). Solvent A consisted of 0.1 % formic acid in water and solvent B consisted of acetonitrile. A linear gradient from 100 % A to 78 % A in 3 min, followed by linear gradient from 78 % A to 55 % A in 7 min and a linear gradient from 55 % A to 0 % A in 2 min was applied. The column was returned to 100 % A in 1 min, after an isocratic elution at 100 % B for 2 min. The samples, 5 µl, were analysed at a flow rate of 0.35 mL/min with a total run time of 15 minutes for each sample. Ionization was achieved with an electrospray source with the cone voltage set at 15 V and capillary voltage at 3 kV. Analysis was carried out utilizing positive and negative modes.

S. frutescens triterpenoids were fractionated on a semi-preparative Novapak HR C18 high pressure liquid chromatography (HPLC) column (6 µm spherical particles, 300 mm×7.8 mm; Millipore-Waters, La Jolla, USA) utilizing an elution gradient from 80% solvent A (0.1% trifluoroacetic acid in analytical quality water, *v*/*v*) and 20% solvent B to 70% solvent B (90% acetonitrile and 10% A, *v*/*v*) and eluted over 23 minutes. The major fractions (fractions 1 and 3) were collected and subjected to further analysis by LC-MS/MS to determine the total ion count in positive and negative electron spray ionization (ESI) modes. The fractions were subjected to the same LC-MS/MS method as described for the methanolic extract above. SUB was collected, lyophilized and dissolved in ethanol, 2 mg/mL, prior to storage at -18 °C.

Preparation of adrenal mitochondrial and microsomal fractions

Microsomal and mitochondrial fractions were prepared by differential centrifugation as described by Yang & Cederbaum (Yang and Cederbaum, 1997). Fresh sheep adrenals were washed with a 15 mM KCl solution after decapsulation and the adrenal tissue (51.36 g) was homogenized in three parts of 10 mM Tris–HCl buffer (pH 7.4) containing 1.0 mM EDTA and 0.25 M sucrose. The homogenate was centrifuged for 15 minutes at 12 000 x g to pellet the mitochondrial fraction. The post-mitochondrial supernatant was centrifuged at 12 000 x g for 15 minutes. To prepare the microsomes, a 50 % (w/v) PEG 8000 solution was added slowly with stirring to the supernatant to a final concentration of 8.5 % and the mixture was stirred for 10 minutes and subsequently centrifuged at 13 000 x g for 20 minutes. The microsomal pellets were homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl and 1 mM

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EDTA. PEG (50 % w/v) was again added to the solution to a final concentration of 8.5 % and the mixture was stirred and centrifuged at 13 000 x g for 20 minutes. This procedure was repeated until the supernatant was clear. The final microsomal pellet was resuspended in 80 mL of 10 mM Tris-HCl buffer (pH7.4) containing 1 mM EDTA and 0.25 M sucrose and stored at -80 ºC until further use. The cytochrome P450 concentrations were determined and protein determination was carried out by using the BCA method (Pierce Biotechnology, USA) as instructed by the manufacturer.

Determination of the mitochondrial cytochrome P450 concentration

The cytochrome P450 concentration of the mitochondrial and microsomal fractions were determined using a method previously described by Omura and Sato (Omura and Sato, 1964). The dried mitochondrial fraction was resuspended in 0.1 M phosphate buffer (pH7.4, 10 % ethylene glycol) to a final concentration of 2 mg/mL. The solution was placed on ice and sonicated with 1 minute intervals for a total of 5 minutes and subsequently saturated with carbon monoxide. The microsomal solution was diluted using 0.1 M phosphate buffer (pH7.4, 10 % ethylene glycol) where after it was saturated with carbon monoxide. One millilitre of either microsomal suspension or mitochondrial sonicate was added to each of two optically matched cuvettes (representative of the sample and reference). After recording a baseline between 400 and 500 nm, an initial spectrum was recorded after the addition of approximately 2 µg of sodium dithionate to the sample cuvette. The spectrum was allowed to develop for approximately 10 minutes, where after a final reading was taken.

The Beer-Lambert law (Equation 1) was used to calculate the P450 concentration using the molar extinction coefficient previously determined by Omura and Sato (Omura and Sato, 1964).

$$\Delta A = \varepsilon c l \qquad \text{Equation 4.1}$$

Where Δ = change in absorbance; ε = Molar extinction coefficient; c = concentration of the solution and l = path length (1 cm)

Spectral binding assays

Spectra were recorded between 385 and 500 nm using a Cary 100 Conc UV/Visible Spectrophotometer (Varian). Type 1 substrate-induced difference spectra were obtained with PROG (3.2 µM) in the microsomal preparations (P450, 0.35 µM) (Prevoo et al 2008) and DOC or deoxycortisol (3.2 µM) in the mitochondrial preparations (P450, 0.8 µM). SUA and B were

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assayed at a final concentration of 10 µM with inhibition being indicated by a reduction in amplitude of the difference spectra. The percentage inhibition was calculated by:

$$\% \text{ inhibition} = \{(A - B)/ A\} \times 100 \qquad \text{Equation 4.2}$$

A = the amplitude between the absorbance at 390 nm and 420 nm without test compound.

B = the amplitude between the absorbance at 390 nm and 420 nm with test compound.

Steroid conversion assays in COS-1 cells

The influence of the methanolic *S. frutescens* extract and SUB on recombinant enzymes, 3β-HSD2, CYP17A1, CYP21A2, CYP11B1 and CYP11B2, was assayed in non-steroidogenic COS-1 cells as described previously (Schloms et al., 2012). Confluent cells were seeded into 12 well plates, 1×10^5 cells/mL/well, 24 hours prior to transfection. Cells were transiently transfected with 0.5 µg DNA and 1.5 µl *Mirus TransIT*®- LT1 transfection reagent according to the manufacturer's instructions. Cells were incubated for 72 hours after which the appropriate steroid substrate, at a final concentration of 1 µM, was added to the culture medium. PREG was added to cells expressing the baboon 3β-HSD2 and CYP17A1, PROG was added to cells expressing CYP17A1 and CYP21A2, deoxycortisol was added to cells expressing CYP11B1 and DOC was added to the cells expressing CYP11B1 and CYP11B2. The influence of the methanol *S. frutescens* extract was determined by adding 2.66 mg/mL extract to the medium. A pCIneo vector containing no insert DNA was used to perform control transfections. Aliquots, 500 µl, were removed after 4 hours (3β-HSD2, CYP17A1 and CYP21A2) and 8 hours (CYP11B1 and CYP11B2) and the steroids extracted using a 10:1 volume of dichloromethane to culture medium. The mixture was vortexed for 30 seconds, centrifuged at 500 × g for 5 minutes and the medium aspirated. The dichloromethane phase containing the steroids was dried under N_2, resuspended in 120 µl methanol and stored at 4 °C prior to steroid analysis by ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS). Metabolites were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Waters UPLC BEH C18 column as previously described (Schloms et al., 2012) and a Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. Calibration curves were constructed by using weighted (1/x2) linear least squares regression. Data was collected with the MassLynx 4.0 software program and steroid analyses presented as the mean, n=6.

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Steroid metabolism in H295R cells

The influence of a methanolic *S. frutescens* extract and SUB was assayed on steroid metabolism in H295R cells as previously described (Schloms et al., 2012). The extract was assayed under basal, forskolin and AngII stimulation, whereas SUB and GABA were assayed under basal conditions only. Briefly, confluent cells were seeded into 12 well plates, $4x10^5$ cells/mL/well, and allowed to attach for 48 hours after which the medium was replaced with growth medium containing 0.1 % cosmic calf serum. Cells were incubated for 12 hours after which extract (1 mg/mL), SUB (10 or 30 µM), SU mixture (10 or 30 µM) or GABA (10 µM) was added, with and without forskolin or AngII in the case of the extract, to determine effects on basal and stimulated steroid production. Aliquots, 500 µl, were collected after 48 hours and the steroids extracted as described in the previous section. The steroid residue was resuspended in 150 µl methanol for analysis by UPLC-LC-MS. Steroids were separated using a Phenomenex UPLC Kinetex PFP (2.1 mm × 100 mm, 2.6 µm) column and analysed and quantified by UPLC-APCI-MS (Schloms et al., 2012) as described above. All experiments were performed either two or three times and tabulated data are representative of at least two experiments. Each experiment was conducted in triplicate and results are presented as means ± SEM.

Transactivation and transrepression assays

The effects of the extract and SUB on the transcriptional activities of the GR were assayed in COS-1 cells which were plated in complete medium (DMEM containing 10% fetal calf serum and 1% penicillin- streptomycin) at a density of $2x10^6$ cells/mL in 10 cm culture dishes. After 24 hours the cells were transiently transfected with the appropriate expression vectors, using a *Trans*IT®-LT1 transfection reagent (Mirus, USA) according to the manufacturer's instructions. For transactivation assays, 900 ng pRS-hGR and 9 µg pTAT-2xGRE-Elb-luciferase, driven by the E1b promoter containing two copies of the rat TAT-GRE (Sui, 1999) were used, while for transrepression assays 1.5 µg pRS-hGR and 3 µg of the IL6-luc-promoter reporter construct (p(IL6κB)350hu.IL6Pluc+) were used. After 24 hours, the transfected cells were seeded into 24 well plates at a density of 5 x 10^4 cells/well and incubated for 24 hours. The cells were washed with phosphate buffered saline (PBS) and incubated for 24 hours with serum-free medium containing dexamethasone (Dex) (10 µM), *S. frutescens* (0.5 or 0.75 mg/mL) and SUB (10 or 30 µM) (for transactivation) or serum-free medium containing PMA in the absence and presence of Dex (10 µM), extract (0.5 or 0.75 mg/mL) and SUB (10 or 30 µM) (for transrepression).

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The effects of the extract and SUB on the MR were assayed in CHO cells stably transfected with the rat MR, 11β-HSD2 and a pTAT3-gLuc reporter gene (Morita et al., 1996). Cells were seeded at a density of 2.5 x 10^4 cells/mL/well in 12 well plates in complete growth media (DMEM/F-12 containing 10% cosmic calf serum, 1% penicillin- streptomycin and 0.1 % gentamicin) and incubated for 48 hours. The cells were incubated for 24 hours in experimental medium (DMEM/F-12 containing 0.1 % charcoal stripped cosmic calf serum, 1% penicillin-streptomycin and 0.1 % gentamicin) containing ALDO (10 nM), *S. frutescens* (0.5 and 0.75 mg/mL) and SUB (10 and 30 µM) to investigate agonist activity, and containing 10 nM ALDO in the absence and presence of *S. frutescens* (0.5 or 0.75 mg/mL), SUB (10 or 30 µM) or SPL (1 µM) to investigate antagonist activity.

Lysed cells were analysed as previously described (Africander et al., 2011) for all the reporter assays. The luciferase activity, measured in relative light units (RLUs), was normalized to protein concentration as determined using a Pierce® BCA Protein Assay Kit (Rockford, IL, USA) to correct for plating efficiency.

Cell viability assays

The viability of the COS-1, H295R and CHO cells in the presence of *S. frutescens* extract and SUB was determined using an *in vitro* cytotoxicity assay kit based on an 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich) according to the manufacturer's instructions. The concentrations of cells used for these assays were $1x10^5$ cells/mL for COS-1 cells, $4x10^5$ cells/mL for H295R cells and $2.5x10^4$ cells/mL for CHO cells. SUB was assayed at 10 and 30 µM and the extract was assayed at 0.5, 0.75, 1 and 2.6 mg/mL. Cell viability was not affected at these concentrations and no detrimental or stimulatory effects were observed. In addition, the 17-dehydrogenase activity endogenous to COS1 cells was not affected by *S. frutescens* with the conversion of testosterone to A4 being ± 90% in the presence of the extract (2.5mg/mL) as previously reported (Sergeant, 2009).

Statistical analysis

Experiments were repeated in duplicate with three replicates within each experiment (unless stated otherwise). Mean values of data obtained in each experiment were analyzed using one-way ANOVA and appropriate post-tests (as indicated in figure legends). The error bars represent the standard error of the mean (SEM) and a value of $P<0.05$ was considered statistically significant. All data manipulations, graphical representations and statistical analysis were performed using Graph Pad Prism 5 (GraphPad Software, Inc, CA, USA).

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Results

The effect of a methanolic extract of *S. frutescens* on adrenal steroidogenic enzymes.

As previous investigations have shown the effects of an aqueous extract on CYP17A1, CYP21A2 and CYP11B1, we investigated a methanolic extract on steroid conversion by CYP17A1, CYP21A2, CYP11B1, 3β-HSD2 and CYP11B2. In addition, we investigated whether the methanolic extract of *S. frutescens* would influence steroid production in H295R cells. These cells were chosen as an experimental model as they mimic steroid production in in the adrenal gland and produce glucocorticoids, mineralocorticoids and adrenal androgens (Rainey et al., 1994; Wang and Rainey, 2012). The effects of the extract on steroid production were thus investigated under both basal and stimulated conditions.

S. frutescens methanolic extracts influence steroid conversion assays in COS-1 cells

Steroid substrate conversion by 3β-HSD2, CYP17A1, CYP21A2, CYP11B1 and CYP11B2 was assayed in the presence of the extract to assess the effect of *S. frutescens* on the catalytic activity of individual recombinant steroidogenic enzymes, in the absence of competing enzymes, in a simple cell model. COS-1 cells were transiently transfected with either baboon 3β-HSD2, baboon CYP17A1, bovine CYP21A2 or co-transfected with either human CYP11B1 and Fedx or CYP11B2 and Fedx. As discussed in chapter 3, Fedx is required for effective electron transfer between FeRed and CYP11B1 or CYP11B2 (Grinberg et al., 2000). Since 3β-HSD2 catalyses the conversion of PREG to PROG, 17OH-PREG to 17OH-PROG and DHEA to A4, the three steroid substrates were added at a final concentration of 1 µM, to cells transiently expressing the enzyme and assayed in the absence and presence of extract (2.66 mg/mL). Figure 4.1 shows that the presence of the methanol extract had no effect on the conversion of PREG to PROG (Fig.4.1A) or the conversion of DHEA to A4 (Fig.4.1C). However, the conversion of 17OH-PREG to 17OH-PROG was inhibited significantly ($P<0.001$), resulting in ± 20 % lower 17OH-PROG levels (Fig.4.1B).

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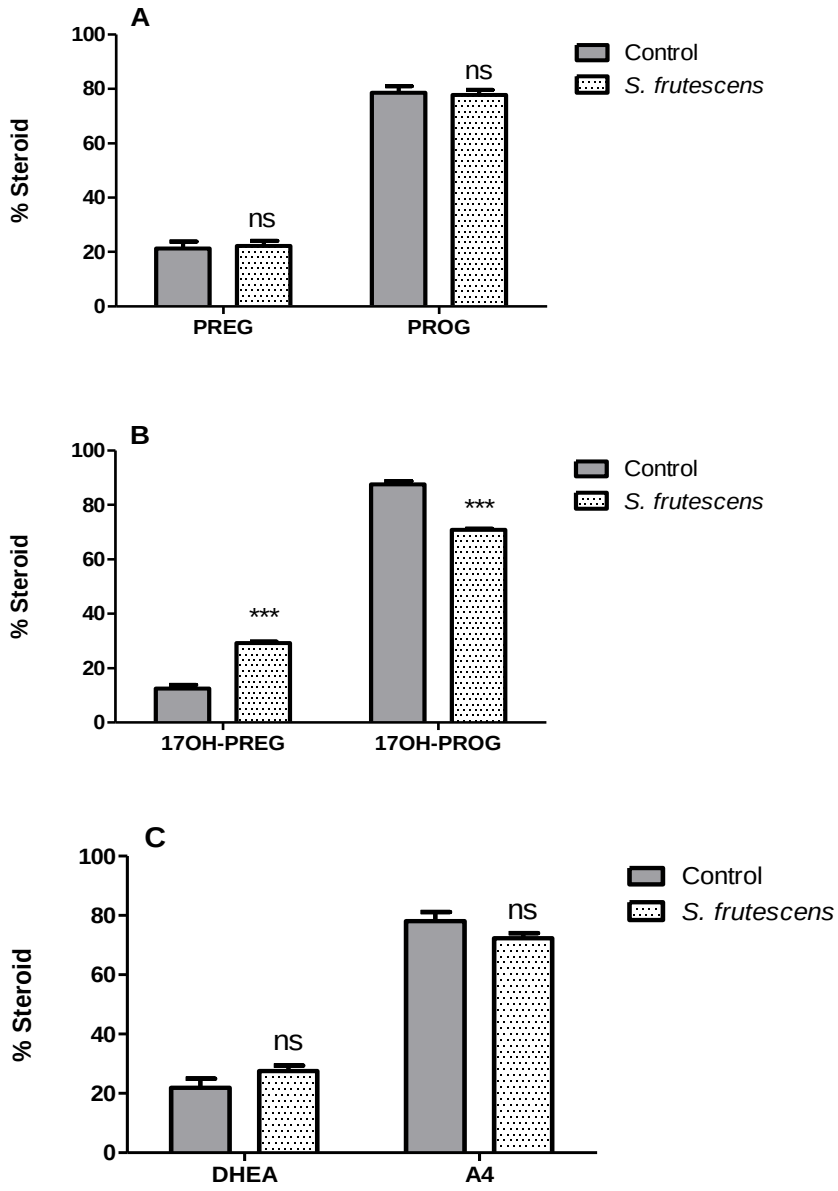

Fig. 4.1: Effect of the methanolic extract of *S. frutescens* on steroid conversion in COS-1 cells expressing baboon 3β-HSD2. A) PREG conversion to PROG, B) 17OH-PREG conversion to 17OH-PROG and C) DHEA conversion to A4, after 4 hours. Steroid substrates, 1 µM; *S. frutescens* extract, 2.66 mg/mL. The data represents the mean of two independent experiments conducted in triplicate (n=6) and statistical analysis was performed using a one-way ANOVA with Dunnett's post-test (***, P<0.001; ns, non-significant).

The effect of the extract on PREG and PROG conversion by CYP17A1 was subsequently assessed. PREG is hydroxylated by CYP17A1 to produce 17OH-PREG, which, in turn, undergoes a lyase reaction to produce DHEA. Similarly, CYP17A1 hydroxylates PROG to produce 17OH-PROG which, since CYP17A1 does not exhibit 20, 21-lyase activity towards 17OH-PROG, is not converted to A4. The presence of the extract appeared to have no

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significant inhibitory effect on either the hydroxylase or lyase catalysed reactions in the conversion of PREG to 17OH-PREG and subsequently to DHEA (Fig. 4.2A), however the conversion of PROG to 17OH-PROG by CYP17A1 was significantly inhibited (± 50 % inhibition), while A4 was not detected after 4 hours as expected (Fig. 4.2B).

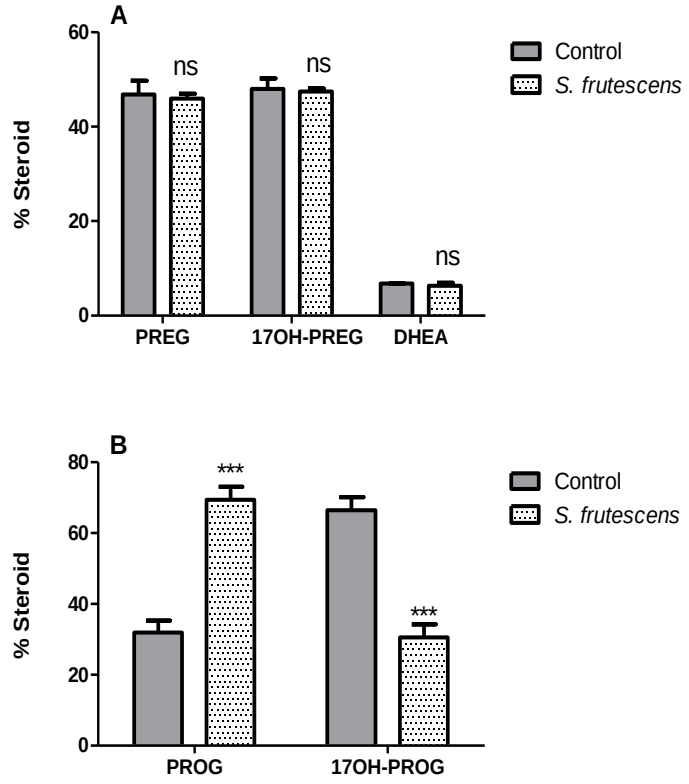

Fig. 4.2: Effect of the methanolic extract of *S. frutescens* on steroid conversion in COS-1 cells expressing baboon CYP17A1. A) PREG conversion to 17OH-PREG and DHEA and B) PROG conversion to 17OH-PROG, after 4 hours. Steroid substrates, 1 µM; *S. frutescens* extract, 2.66 mg/mL. The data represents the mean of two independent experiments conducted in triplicate (n=6) and statistical analysis was performed using a one-way ANOVA with Dunnett's post-test (***, P<0.001; ns, non-significant).

As CYP17A1 is not the only P450 enzyme that metabolises PROG, with CYP21A2 also catalysing the conversion of the steroid, the effect of the extract on the catalytic activity of CYP21A2 was also investigated. *S. frutescens* inhibited the conversion of PROG to DOC significantly with ± 87 % substrate remaining after 4 hours (Fig. 4.3).

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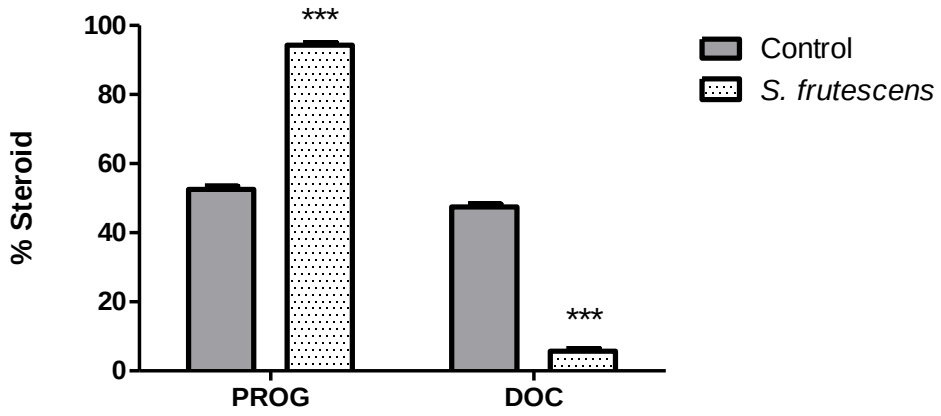

Fig. 4.3: Effect of the methanol extract of *S. frutescens* on steroid conversion in COS-1 cells expressing bovine CYP21A2. The figure shows PROG conversion to DOC by CYP21A2 after 4 hours in the absence and presence of the extract. PROG, 1 µM; *S. frutescens* extract, 2.66 mg/mL. The data represents the mean of two independent experiments conducted in triplicate (n=6) and statistical analysis was performed using a one-way ANOVA with Dunnett's post-test (***, P<0.001).

The effect of *S. frutescens* on the catalytic activity of CYP11B1 and CYP11B2, the enzymes catalysing the final steps in adrenal glucocorticoid and mineralocorticoid biosynthesis, was subsequently assessed. The conversion of both DOC and deoxycortisol by CYP11B1 (Fig. 4.4) and DOC by CYP11B2 (Fig. 4.5) were determined in the absence and presence of the extract. Both enzymes were co-transfected with Fedx as it is required for effective electron transfer between the endogenous reductase and CYP11B1 or CYP11B2. The conversion of DOC and deoxycortisol to CORT (Fig. 4.4 A) and cortisol (Fig. 4.4 B) was significantly inhibited in the presence of the extract, with the inhibition being similar for both substrates. A greater inhibitory effect was observed in the conversion of DOC by CYP11B2 in the presence of *S. frutescens* (Fig 4.5). The conversion of DOC to CORT in the initial hydroxylation at C11 in the production of ALDO was inhibited significantly. The inhibitory effect of *S. frutescens* on the subsequent 18-hydroxylase and 18-oxidase activities in the production of 18OH-CORT and ALDO was non-significant. This can be ascribed to more than 50% DOC remaining after 8 hours and, together with CORT levels being reduced 1.9-fold, resulted in a 1.3-fold decrease in the 18OH-CORT intermediate and a 1.6-fold decrease in ALDO production.

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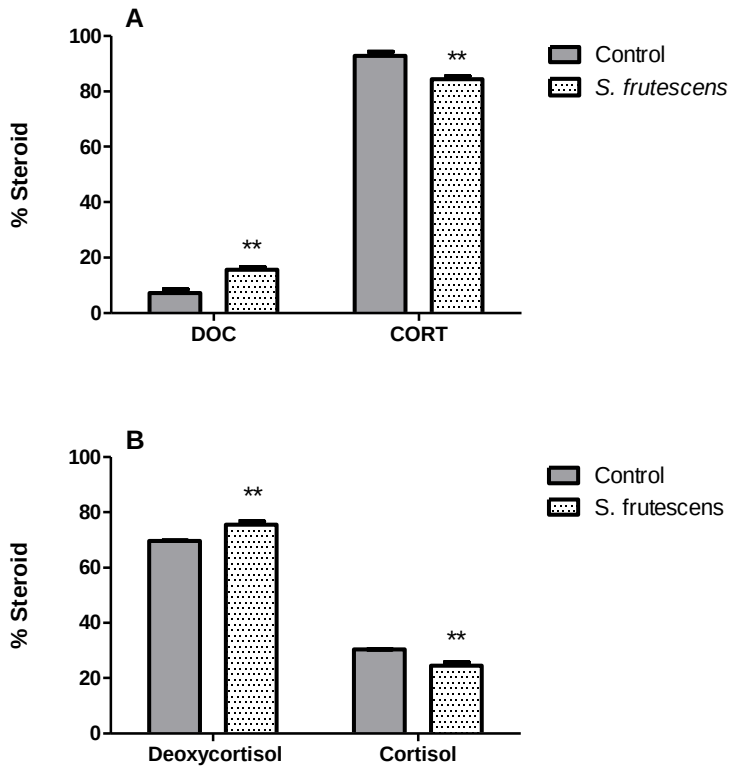

Fig. 4.4: Effect of the methanol extract of *S. frutescens* on steroid conversion in COS-1 cells expressing bovine human CYP11B1 and human Fedx. The figure shows A) DOC conversion to CORT and B) deoxycortisol conversion to cortisol, after 8 hours. Steroid substrates, 1 µM; *S. frutescens* extract, 2.66 mg/mL. The data represents the mean of two independent experiments conducted in triplicate (n=6) and statistical analysis was performed using a one-way ANOVA with Dunnett's post-test (**, $P<0.01$).

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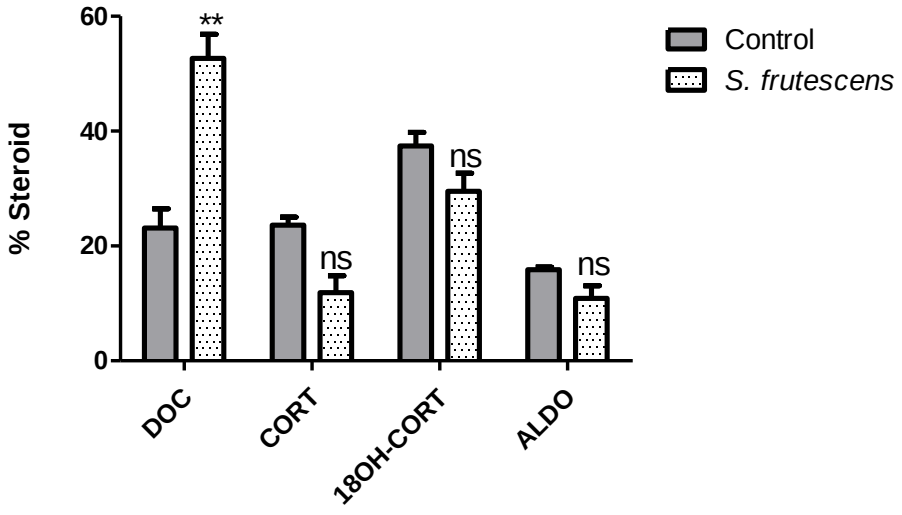

Fig. 4.5: Effect of the methanol extract of *S. frutescens* on steroid conversion in COS-1 cells expressing human CYP11B2 and Fedx after 8 hours. DOC, 1 µM; *S. frutescens* extract, 2.66 mg/mL. The data represents the mean of two independent experiments conducted in triplicate (n=6) and statistical analysis was performed using a one-way ANOVA with Dunnett's post-test (**, P<0.01; ns, non-significant).

In summary, *S. frutescens* inhibited 3β-HSD2 activity in a substrate specific manner in which the conversion of 17OH-PREG was affected, the hydroxylation of PROG by both CYP17A1 and CYP21A1 was inhibited, while the conversion of PREG by CYP17A1 was not. Both CYP11B1 and CYP11B2 were affected by *S. frutescens* with data suggesting a greater inhibition of CYP11B2.

Steroid metabolism in H295R cells

The results obtained in the experiments in COS-1 cell model and in previous investigations clearly show that the methanolic extract of *S. frutescens* does affect substrate conversion by individual steroidogenic enzymes. The effect of the *S. frutescens* extract on steroidogenesis was subsequently assayed in a more complex cell model expressing adrenal steroidogenic enzymes to determine the manner in which steroid production in the glucocorticoid, mineralocorticoid and androgen pathways would be affected.

Methanolic *S. frutescens* extracts alter basal steroid production in H295R cells

H295R cells were incubated in the absence and presence of the extract to firstly determine the effect on basal steroid production (Table 4.1). The production of CORT (± 250 to 125 nM), 16OH-PROG (± 50 to 35 nM), deoxycortisol (± 1440 to 1070 nM) and cortisol (± 930 to 510 nM) were significantly decreased by the presence of the extract, while DOC (± 6 to 15 nM)

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and DHEAS (± 10 to 25 nM) production increased. Although not statistically significant, total steroid production in the presence of the extract showed a decreasing trend.

The detection limits for both PREG and DHEA, which could not be accurately detected in these experiments, was 100 ng/mL as was previously shown (Schloms et al., 2012).

Table 4.1: Steroid production in H295R cells in the presence of *S. frutescens* extract after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	**Basal**			***S. frutescens* 1 mg/mL**			
	% of Total ± SEM			**% of Total ± SEM**			
PREG	ND			ND			
PROG	0.00	±	0.00	0.00	±	0.00	ns
DOC	0.15	±	0.00	0.50	±	0.05	↑ P<0.01
CORT	6.30	±	0.60	4.30	±	0.60	↓ P<0.001
18OH-CORT	0.35	±	0.10	0.40	±	0.10	ns
ALDO	0.05	±	0.05	0.05	±	0.00	ns
11-DHC	0.70	±	0.05	1.00	±	0.15	ns
17OH-PREG	0.00	±	0.00	0.00	±	0.00	ns
17OH-PROG	1.15	±	0.05	1.65	±	0.10	ns
16OH-PROG	1.20	±	0.00	1.20	±	0.05	↓ P<0.01
Deoxycortisol	35.80	±	1.76	37.00	±	1.10	↓ P<0.01
Cortisol	23.10	±	0.75	17.60	±	6.00	↓ P<0.001
Cortisone	0.20	±	0.05	0.25	±	0.05	ns
DHEA	ND			ND			
DHEAS	0.25	±	0.00	0.85	±	0.15	↑ P<0.01
A4	24.60	±	0.40	28.80	±	0.70	ns
11OH-A4	4.85	±	0.30	5.50	±	0.50	ns
Testosterone	1.30	±	0.5	1.25	±	0.10	ns
Total steroid (nM)	4020.00	±	90.00	2900.00	±	135.5	ns

Forskolin-stimulated steroid production in H295R cells is influenced by *S. frutescens*

The effect of the extract was subsequently investigated in H295R cells under forskolin-stimulated conditions. Shown in Table 4.2 are the effects of forskolin on basal steroid production in the cell model, corroborating findings from previous studies (Schloms et al., 2012; Xing et al., 2011). Results showed that the glucocorticoids and glucocorticoid precursors were significantly increased in the presence of forskolin. The levels of CORT (± 250 to 1800 nM), 18OH-CORT (± 15 to 105 nM), ALDO (± 2 to 30 nM), 11-DHC (± 30 to 60 nM), cortisol (± 930 to 4480 nM), A4 (± 990 to 1100 nM) and 11OHA4 (± 195 to 960 nM) were significantly

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increased after forskolin stimulation. 17OH-PREG and DHEA were not detected under basal conditions, however, the concentrations of these metabolites were increased after forskolin stimulation to ± 95 nM and ± 135 nM, respectively. In addition, steroid output increased significantly with forskolin stimulation with total steroid production (± 11 µM) being greater than that produced under basal conditions (± 4 µM).

Table 4.2: Steroid production in H295R cells in the presence of forskolin after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	**Basal**	**10 µM Forskolin**	
	% of Total ± SEM	**% of Total ± SEM**	
PREG	ND	ND	ns
PROG	0.02 ± 0.00	0.01 ± 0.00	ns
DOC	0.17 ± 0.02	0.10 ± 0.01	ns
CORT	6.31 ± 0.60	16.73 ± 0.41	↑ P<0.001
18OH-CORT	0.33 ± 0.11	0.96 ± 0.03	↑ P<0.001
ALDO	0.07 ± 0.04	0.29 ± 0.10	↑ P<0.01
11-DHC	0.69 ± 0.03	0.54 ± 0.14	↑ P<0.001
17OH-PREG	ND	0.87 ± 0.20	↑ P<0.001
17OH-PROG	1.13 ± 0.06	0.24 ± 0.03	ns
16OH-PROG	1.19 ± 0.02	0.39 ± 0.01	ns
Deoxycortisol	35.79 ± 1.76	10.86 ± 2.02	ns
Cortisol	23.12 ± 0.75	41.54 ± 3.42	↑ P<0.001
Cortisone	0.18 ± 0.03	0.11 ± 0.01	ns
DHEA	ND	1.24 ± 0.46	↑ P<0.001
DHEAS	0.23 ± 0.02	0.11 ± 0.01	ns
A4	24.61 ± 0.40	10.25 ± 1.62	↑ P<0.001
11OHA4	4.84 ± 0.30	8.92 ± 1.07	↑ P<0.001
Testosterone	1.31 ± 0.06	0.38 ± 0.07	ns
Total steroid (nM)	4020 ± 90	10790 ± 720	↑ P<0.001

As forskolin represents the stress response in the adrenal cell, the data generated were firstly compared to basal steroid production to allow comparative analyses of the effects of *S. frutescens* on steroid production under stimulated conditions with basal levels (Table 4.3). Most of the steroid metabolites in the three pathways were significantly increased — DOC (± 6 to 10 nM), CORT (± 250 to 750 nM), 18OH-CORT (± 15 to 65 nM), ALDO (± 2 to 18 nM), 11-DHC (± 30 to 85 nM), cortisol (± 930 to 2050 nM) and 11OHA4 (± 195 to 395 nM). Under basal conditions 17OH-PREG and DHEA were not detected, however after foskolin stimulation in the presence of the extract these metabolites were detected at ± 90 nM and 130 nM, respectively. Evident from these analyses is that the extract did not attenuate the effects of

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forskolin back to basal steroid levels. Steroid production in stimulated cells in the presence of extract was significantly greater than in unstimulated cells (± 6.6 µM vs 4 µM). In addition, while deoxycortisol and A4 levels were comparable to the basal levels, 17OH-PROG and 16OH-PROG were lower than basal levels, 2-fold and 1.5-fold, respectively. It is interesting to note that testosterone was also lower (± 50 to 35 nM) than basal levels.

Table 4.3: Steroid production in H295R cells in the presence and absence of forskolin and 1 mg/mL *S. frutescens* extract after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	**Basal**	**10 µM Forskolin+ *S. frutescens***	
	% of Total ± SEM	**% of Total ± SEM**	
PREG	ND	ND	ns
PROG	0.02 ± 0.00	0.03 ± 0.01	ns
DOC	0.17 ± 0.02	0.17 ± 0.03	↑ P<0.001
CORT	6.31 ± 0.60	11.32 ± 0.30	↑ P<0.001
18OH-CORT	0.33 ± 0.11	1.02 ± 0.07	↑ P<0.001
ALDO	0.07 ± 0.04	0.28 ± 0.04	↑ P<0.05
11-DHC	0.69 ± 0.03	1.28 ± 0.25	↑ P<0.001
17OH-PREG	ND	1.36 ± 0.06	↑ P<0.001
17OH-PROG	1.13 ± 0.06	0.34 ± 0.02	ns
16OH-PROG	1.19 ± 0.02	0.49 ± 0.01	ns
Deoxycortisol	35.79 ± 1.76	20.70 ± 2.23	ns
Cortisol	23.12 ± 0.75	31.05 ± 2.07	↑ P<0.001
Cortisone	0.18 ± 0.03	0.07 ± 0.01	ns
DHEA	ND	1.95 ± 0.74	↑ P<0.001
DHEAS	0.23 ± 0.02	0.50 ± 0.11	↑ P<0.001
A4	24.61 ± 0.40	14.49 ± 0.80	ns
11OHA4	4.84 ± 0.30	5.96 ± 0.61	↑ P<0.001
Testosterone	1.31 ± 0.06	0.54 ± 0.03	ns
Total steroid (nM)	4020 ± 90	6600 ± 226	↑ P<0.001

When comparing the steroid metabolites produced under forskolin stimulated conditions and in the presence of the extract and forskolin (Table 4.4), it is apparent that the extract inhibited the stimulatory effects of forskolin significantly with steroid production in the presence of the extract decreasing from 10.8 to 6.6 µM. The presence of the extract under stimulated conditions significantly decreased CORT (± 1800 to 750 nM), 17OH-PREG (± 95 to 90 nM), 16OH-PROG (± 40 to 30 nM), cortisol (± 4480 to 2050 nM), A4 (± 1105 to 955 nM) and 11OHA4 (± 960 to 395 nM). However, 11-DHC (± 60 to 85 nM), deoxycortisol (± 1170 to 1370 nM) and DHEAS (± 10 to 30 nM) production was significantly increased in the presence of the extract. These results show that the addition of the extract under forskolin stimulation results

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in a similar inhibitory trend as was observed when basal steroid metabolism was assayed in the presence of the extract — steroid production in the mineralocorticoid pathway is decreased the most (± 2010 to 930 nM), followed by the steroid production in the glucocorticoid (± 5830 to 3565 nM) and androgen pathways (± 2120 to 1410 nM).

Table 4.4: Steroid production in H295R cells in the presence of forskolin and 1 mg/mL *S. frutescens* extract after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	**10 µM Forskolin**	**10 µM Forskolin + *S. frutescens***	
	% of Total ± SEM	**% of Total ± SEM**	
PREG	ND	ND	ns
PROG	0.01 ± 0.00	0.03 ± 0.01	ns
DOC	0.10 ± 0.01	0.17 ± 0.03	ns
CORT	16.73 ± 0.41	11.32 ± 0.30	↓ P<0.001
18OH-CORT	0.96 ± 0.03	1.02 ± 0.07	ns
ALDO	0.29 ± 0.10	0.28 ± 0.04	ns
11-DHC	0.54 ± 0.14	1.28 ± 0.25	↑ P<0.05
17OH-PREG	0.87 ± 0.20	1.36 ± 0.06	↓ P<0.05
17OH-PROG	0.24 ± 0.03	0.34 ± 0.02	ns
16OH-PROG	0.39 ± 0.01	0.49 ± 0.01	↓ P<0.01
Deoxycortisol	10.86 ± 2.02	20.70 ± 2.23	↑ P<0.01
Cortisol	41.54 ± 3.42	31.05 ± 2.07	↓ P<0.05
Cortisone	0.11 ± 0.01	0.07 ± 0.01	ns
DHEA	1.24 ± 0.46	1.95 ± 0.74	ns
DHEAS	0.11 ± 0.01	0.50 ± 0.11	↑ P<0.01
A4	10.25 ± 1.62	14.49 ± 0.80	↓ P<0.05
11OHA4	8.92 ± 1.07	5.96 ± 0.61	↓ P<0.05
Testosterone	0.38 ± 0.07	0.54 ± 0.03	ns
Total steroid (nM)	10790 ± 720	6600 ± 226	↓ P<0.001

AngII-stimulated steroid production is altered in the presence of *S. frutescens* extracts

The data obtained when investigating the effect of *S. frutescens* on basal steroid production showed possible modulatory effects of the mineralocorticoid pathway in that DOC levels were significantly increased while CORT were significantly decreased which may possibly indicate that ALDO production may be affected by *S. frutescens*. Therefore, with the focus thus on the mineralocorticoid pathway, the effect of *S. frutescens* on the stimulation of mineralocorticoid production by AngII was investigated. Table 4.5 shows that even though total steroid production was marginally higher and 18OH-CORT and ALDO levels did not change

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significantly after stimulation, the precursor steroid, CORT, increased significantly (± 55 to 100 nM) after 48 hours in the presence of 10 nM AngII, while basal and AngII-stimulated DOC levels remained comparable. This finding therefore enabled the subsequent analyses of the effects of the extract on steroid flux in the mineralocorticoid pathway, and specifically on the production of DOC and CORT, as ALDO levels remained low, even upon stimulation. In addition, the investigation allowed the assessment of the effects of *S. frutescens* on the stimulatory physiological function that AngII has on an adrenal cell.

Table 4.5: Steroid production in H295R cells in the presence of Ang II after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	Basal	10 nM AngII	
	% of Total ± SEM	% of Total ± SEM	
PREG	ND	ND	ns
PROG	0.00 ± 0.00	0.01 ± 0.01	ns
DOC	0.53 ± 0.05	0.48 ± 0.03	ns
CORT	1.30 ± 0.16	2.26 ± 0.27	↑ P<0.01
18OH-CORT	0.06 ± 0.02	0.01 ± 0.01	ns
ALDO	0.04 ± 0.02	0.04 ± 0.01	ns
11-DHC	0.14 ± 0.04	0.14 ± 0.03	ns
17OH-PREG	0.55 ± 0.38	1.65 ± 0.07	ns
17OH-PROG	0.65 ± 0.01	0.63 ± 0.08	ns
16OH-PROG	1.06 ± 0.04	1.07 ± 0.10	ns
Deoxycortisol	52.44 ± 0.82	49.27 ± 0.44	ns
Cortisol	12.93 ± 0.30	14.28 ± 0.68	ns
Cortisone	0.27 ± 0.04	0.21 ± 0.06	ns
DHEA	ND	ND	ns
DHEAS	0.28 ± 0.04	0.31 ± 0.05	ns
A4	26.72 ± 0.52	25.82 ± 0.34	ns
11OHA4	1.79 ± 0.09	2.22 ± 0.09	ns
Testosterone	1.16 ± 0.08	1.12 ± 0.02	ns
Total steroid (nM)	4365 ± 724	4528 ± 533	ns

The addition of *S. frutescens* to the AngII stimulated H295R cells (Table 4.6) resulted in a reduction of CORT (± 60 to 15 nM), 16OH-PROG (± 45 to 20 nM), deoxycortisol (± 2290 to 640 nM), cortisol (± 565 to 80 nM), A4 (± 1170 to 415 nM), DHEAS (± 12 to 3 nM) and testosterone (± 50 to 10 nM) production when compared to basal steroid production. However, DOC (± 25 to 66 nM), 17OH-PROG (± 30 to 80 nM) and PROG (not detected at first, but increased to 13 nM) levels were significantly higher in the presence of the extract. In the mineralocorticoid pathway, although not significant, 18OH-CORT (66%) and ALDO (50%)

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appeared to be decreased in the presence of *S. frutescens*. However, the upstream 4.4-fold reduction in CORT together with the increased DOC (2.8-fold) and PROG is indicative of a decreased flux in the pathway.

Table 4.6: Steroid production in H295R cells in the presence and absence of AngII and 1 mg/mL *S. frutescens* extract after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	Basal	10 nM Ang II + *S. frutescens*	
	% of Total ± SEM	% of Total ± SEM	
PREG	ND	ND	ns
PROG	0.00 ± 0.00	0.92 ± 0.19	↑ P<0.05
DOC	0.53 ± 0.05	4.64 ± 0.61	↑ P<0.05
CORT	1.30 ± 0.16	0.90 ± 0.04	↓ P<0.01
18OH-CORT	0.06 ± 0.02	0.04 ± 0.04	ns
ALDO	0.04 ± 0.02	0.08 ± 0.05	ns
11-DHC	0.14 ± 0.04	0.63 ± 0.08	ns
17OH-PREG	0.55 ± 0.38	2.40 ± 1.28	ns
17OH-PROG	0.65 ± 0.01	5.39 ± 0.30	↑ P<0.01
16OH-PROG	1.06 ± 0.04	1.33 ± 0.09	↓ P<0.05
Deoxycortisol	52.44 ± 0.82	44.90 ± 0.97	↓ P<0.05
Cortisol	12.93 ± 0.30	5.46 ± 1.20	↓ P<0.01
Cortisone	0.27 ± 0.04	0.37 ± 0.05	ns
DHEA	ND	ND	ns
DHEAS	0.28 ± 0.04	0.23 ± 0.02	↓ P<0.05
A4	26.72 ± 0.52	29.13 ± 0.87	↓ P<0.05
11OHA4	1.79 ± 0.09	2.22 ± 0.29	ns
Testosterone	1.16 ± 0.08	0.60 ± 0.15	↓ P<0.05
Total steroid (nM)	4365 ± 724	1426 ± 141	↓ P<0.05

The effect of the extract on AngII stimulated steroid production was investigated (Table 4.7) and the results showed that the extract significantly decreased total steroid production after AngII stimulation (± 4.5 to 1.4 µM). In the presence of the extract, the concentrations of PROG (± 1 to 13 nM), DOC (± 25 to 65 nM) and 17OH-PROG (± 30 to 75 nM) were significantly increased in the AngII stimulated cells. Conversely, CORT (± 100 to 15 nM), 16OH-PROG (50 to 20 nM), deoxycortisol (± 230 to 640 nM), cortisol (± 650 to 80 nM), A4 (± 1170 to 415 nM), 11OHA4 (± 100 to 30 nM) and testosterone (50 to 10 nM) concentrations were significantly decreased in stimulated cells in the presence of the extract. Interestingly, the steroid flux through the glucocorticoid pathway was inhibited to the greatest extent (± 70%), followed by the steroid production in the androgen (± 66%) and mineralocorticoid pathway (±

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30%). The extract had no significant effect on DHEAS production in the AngII stimulated cells, however, DHEAS levels appeared to be reduced.

Table 4.7: Steroid production in H295R cells in the presence of AngII and 1mg/mL *S. frutescens* extract after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	**10 nM AngII**	**10 nM AngII + *S. frutescens***	
	% of Total ± SEM	**% of Total ± SEM**	
PREG	ND	ND	
PROG	0.01 ± 0.01	0.92 ± 0.19	↑ P<0.05
DOC	0.48 ± 0.03	4.64 ± 0.62	↑ P<0.05
CORT	2.26 ± 0.27	0.90 ± 0.04	↓ P<0.001
18OH-CORT	0.01 ± 0.01	0.04 ± 0.04	ns
ALDO	0.04 ± 0.01	0.08 ± 0.05	ns
11-DHC	0.14 ± 0.03	0.63 ± 0.08	ns
17OH-PREG	1.65 ± 0.07	2.40 ± 1.28	ns
17OH-PROG	0.63 ± 0.08	5.39 ± 0.30	↑ P<0.01
16OH-PROG	1.07 ± 0.10	1.33 ± 0.09	↓ P<0.05
Deoxycortisol	49.27 ± 0.44	44.90 ± 0.93	↓ P<0.05
Cortisol	14.28 ± 0.68	5.46 ± 1.18	↓ P<0.001
Cortisone	0.21 ± 0.06	0.37 ± 0.05	ns
DHEA	ND	ND	
DHEAS	0.31 ± 0.05	0.23 ± 0.02	ns
A4	25.82 ± 0.34	29.13 ± 0.75	↓ P<0.05
11OHA4	2.22 ± 0.09	2.22 ± 0.29	↓ P<0.05
Testosterone	1.12 ± 0.02	0.60 ± 0.15	↓ P<0.05
Total steroid (nM)	4528 ± 533	1426 ± 141	↓ P<0.05

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The effect of SUB, one of the major triterpenoid in *S. frutescens* extracts, on adrenal steroidogenic enzymes and overall steroidogenesis.

The results in the previous experiments showed that the methanolic extract of *S. frutescens* influences glucocorticoid and mineralocorticoid production under basal and stimulated conditions. We hypothesized that SUB may be one of the compounds within the extract that contributes significantly to these effects and subsequently investigated the effects of SUB on steroidogenesis in a single enzyme expression system and the influence of SUB on adrenal steroid production in H295R cells. The effect of SUB on steroid substrate binding was also considered in this study, together with SUA. However, studies using SUA were hampered as limited amounts were purified, sufficient for binding assays only.

Analysis of the methanolic extract of *S. frutescens* and SU compounds

The *S. frutescens* extract was analysed in both ESI negative and positive modes, with a clearer visualization of the sutherlandins and SU compounds being obtained in ESI negative mode (Fig. 4.6), depicted in the total ion chromatogram (TIC).

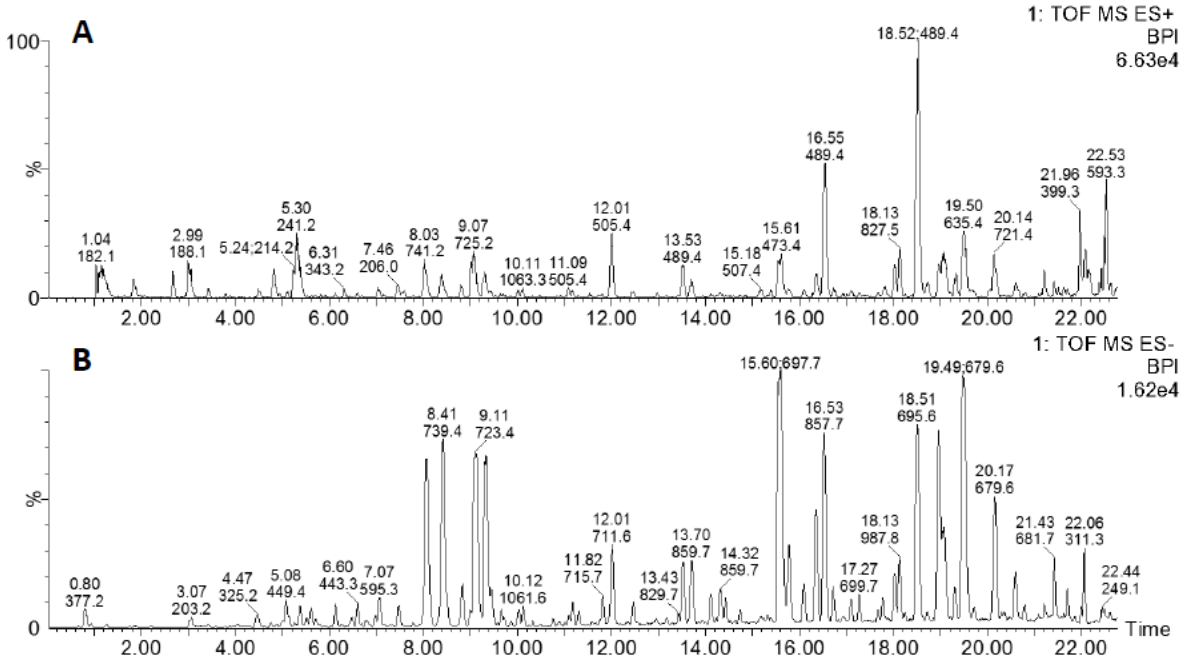

Fig. 4.6. Total ion chromatogram of the LC-MS analysis of a methanolic extract of *S. frutescens* in A) ESI^+ and B) ESI^- modes. The major peaks visible in B) are representative of the sutherlandins and sutherlandiosides.

A crude mixture of SUs was obtained and fractionated using semi-preparative HPLC. The SU compounds exhibited a similar profile as previously reported, eluting in the order of SUB, SUC, SUA and SUD (Fig.4.7).

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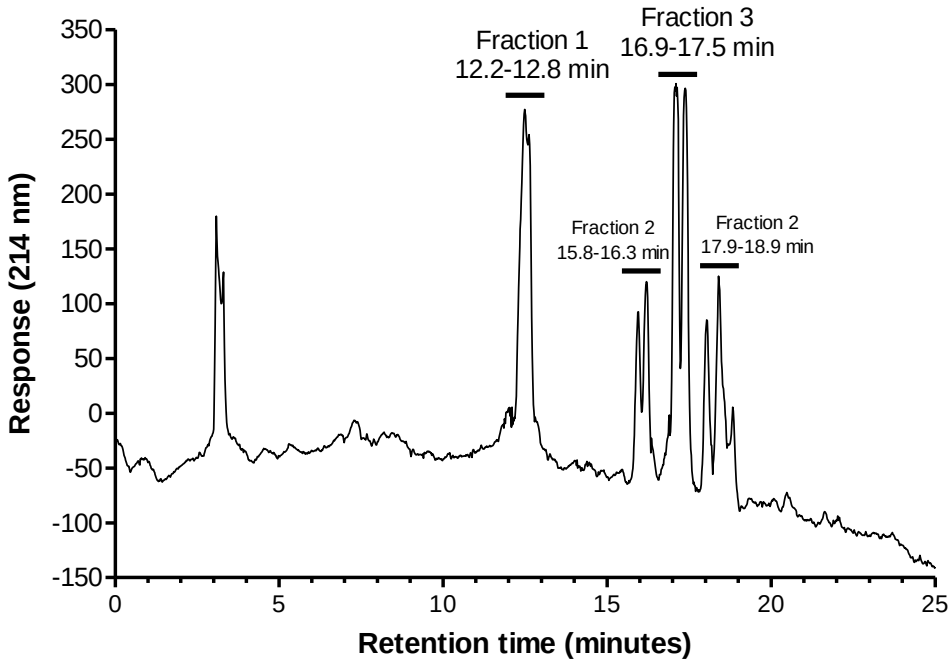

Fig. 4.7: A representative semi-preparative HPLC chromatogram of the crude sutherlandiosides mixture chromatographed on a C18 matrix. The collection window in terms of HPLC retention times of the two major fractions (fractions 1 and 3) and minor fractions (fractions 2 and 4) are given above the relevant high absorbing peaks.

Fraction 1 and 3 were collected upon fractionation of the mixture of SU compounds and analysed by LC-MS/MS in ESI negative and positive modes with the resulting ions correlating to that previously described (Albrecht et al., 2012). Fraction 1 was identified as SUB and fraction 3 was identified as SUA (structures shown in Fig. 4.8).

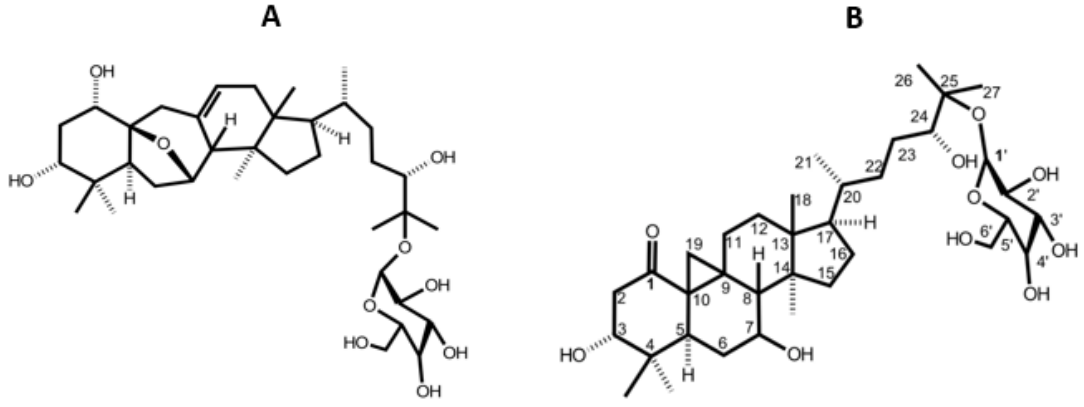

Fig 4.8. Chemical structures of SUA (A) and SUB (B) as determined by Fu et al. (Fu et al., 2008).

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Both compounds have the same exact mass, 652.41, and similar structures, and the fragmentation patterns of SUA and SUB (Fig. 4.9) are also identical with the following extracted ions being identified in ESI positive mode m/z 653.4281 $[M+H]^+$; m/z 635.4281 $[M+H-H2O]^+$; m/z 491.3741 $[M+H-glu]^+$; m/z 473.3635 $[M+H-glu-H2O]^+$; m/z 455.3532 $[M+H-glu-2H2O]^+$; m/z 437.3417 $[M+H-glu-3H2O]^+$ and m/z 419.3275 $[M+H-glu- 4H2O]^+$. In ESI negative mode the major ions previously reported for SUA and B were m/z 651.4080 $[M-H]^-$ and m/z 697.4161 $[M+formate]^-$, respectively (Albrecht et al., 2012). However, in our study m/z 697.4161 $[M+formate]^-$ was detected, but not m/z 651.4080 $[M-H]^-$. Quantification of the major SU compounds in the methanol extract showed SUB was present at 3 mg/g *S. frutescens* dried plant material and SUA, SUC and SUD at 2.33, 1.86 and 2.60 mg/g, respectively. We subsequently focused our bioactivity studies on SUA and SUB and determined their effects on the substrate binding activity of the steroidogenic enzymes in microsomal and mitochondrial preparation.

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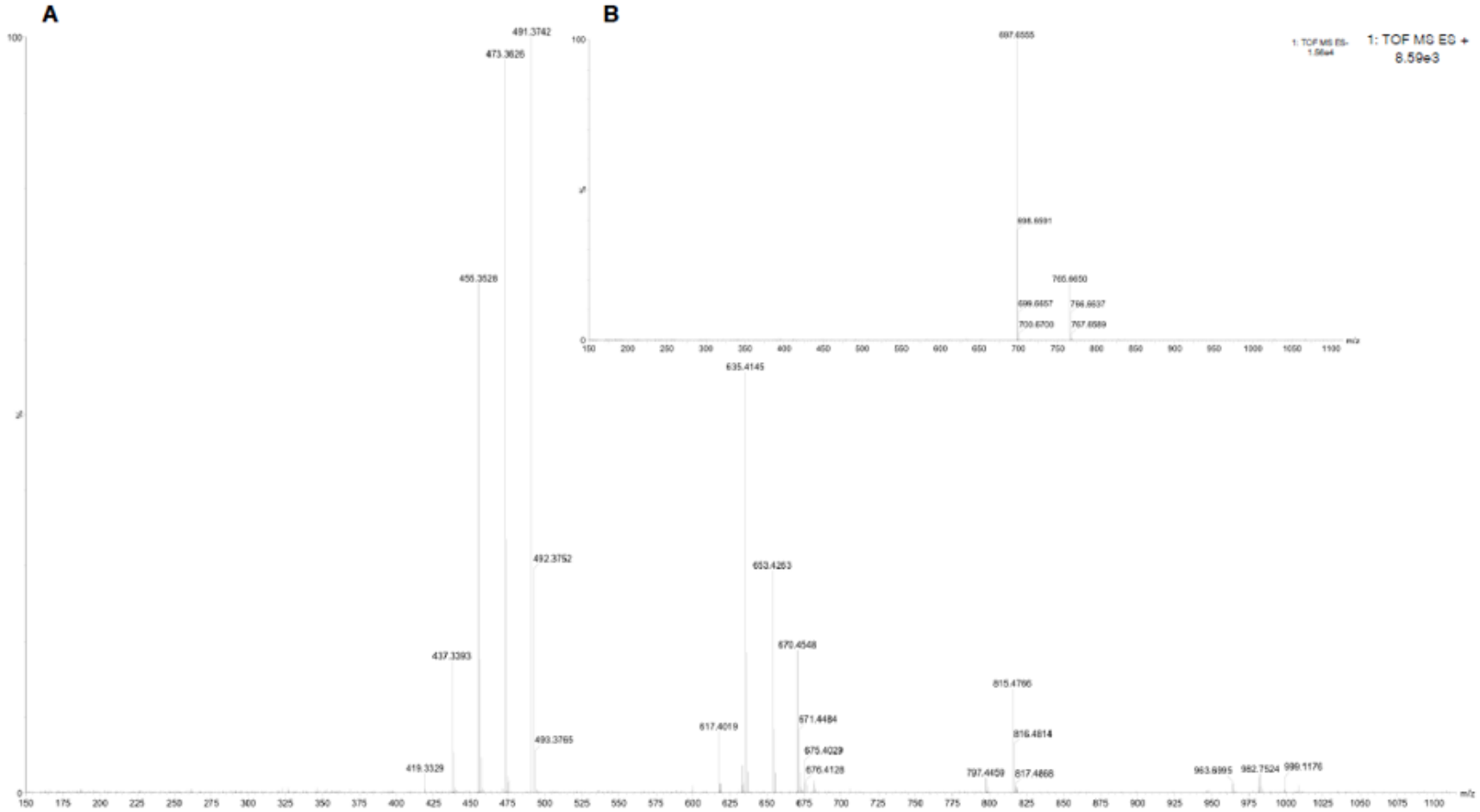

Fig. 4.9. LC-MS chromatogram of SUB in (A) ESI^+ mode and (B) ESI^- mode and SUB structure (insert). In ESI^+ mode m/z 635.4281 $[M+H-H_2O]^+$; m/z 491.3741 $[M+H\text{-}glu]^+$; m/z 473.3635 $[M+H\text{-}glu\text{-}H_2O]^+$; m/z 455.3532 $[M+H\text{-}glu\text{-}2H_2O]^+$; m/z 437.3417 $[M+H\text{-}glu\text{-}3H_2O]^+$ and m/z 419.3275 $[M+H\text{-}glu\text{-}4H_2O]^+$ were detected. In ESI^- mode the major ions detected was m/z 697.4161 $[M+formate]^-$ 653.4281 $[M+H]^+$.

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Substrate binding to P450 enzymes is inhibited by SUB

Previous studies have shown that a triterpenoid fraction isolated from *S. frutescens* inhibited substrate binding and conversion in adrenal microsomal preparations (Prevoo et al. 2008). The biological activity of SUA and B was therefore determined by investigating the ability of these compounds to interact with steroidogenic P450 enzymes. Microsomal preparations which contain both CYP17A1 and CYP21A2 were incubated with the compounds and PROG. The typical type 1 substrate-induced difference spectra obtained upon addition of PROG to the enzymes is shown in Fig. 4.10. Although the inhibition of the binding of PROG by both SUA and SUB, at 10 µM was significant ($P<0.05$), it was not as prominent as the inhibition of the binding of glucocorticoid precursors to CYP11B1 and CYP11B2. Interestingly, in mitochondrial preparations, which contain CYP11A1, CYP11B1 and CYP11B2, the binding of DOC to the enzymes was inhibited to a greater degree by SUB (70 %) than by SUA (40 %) (Fig. 4.11). In contrast, neither SUB nor SUA inhibited the binding of deoxycortisol to P450 enzymes significantly (Fig. 4.12). As SUB appeared to be the more biologically active SU and due to limited availability of SUA, we conducted further investigations with SUB only.

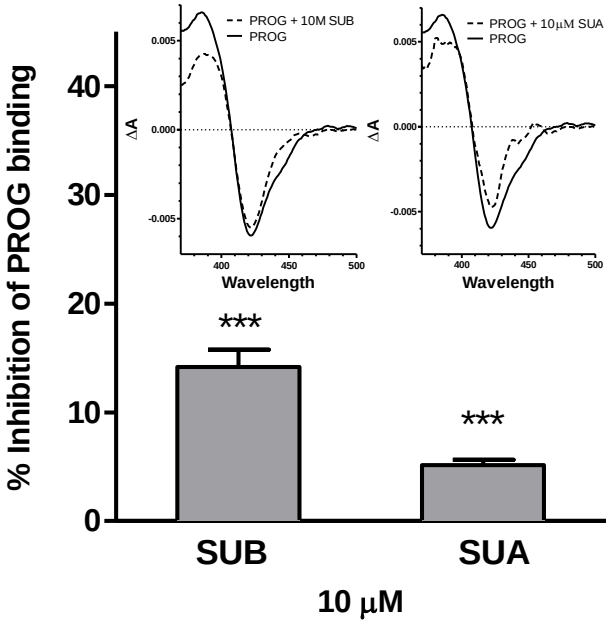

Fig. 4.10: Inhibition of PROG binding in ovine adrenal microsomes in the presence of 10 µM SUB or SUA. [P450 microsomes], 0.35 µM; [PROG], 3.2 µM. Results are presented as the mean, error bars represent SEM and n=3. Inhibition was compared for each concentration using one-way analysis of variance followed by Tukey comparison test (***, $P<0.001$).

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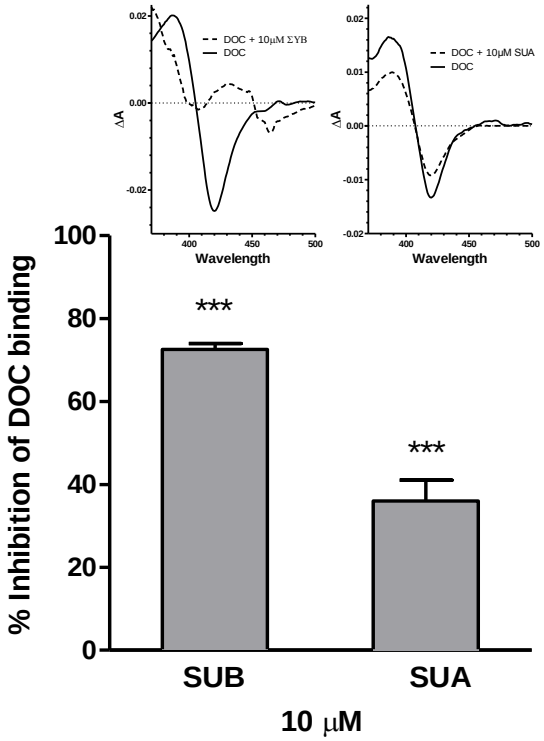

Fig. 4.11: Inhibition of DOC binding in ovine adrenal mitochondria in the presence of 10 µM SUB or SUA. [P450 mitochondria], 0.80 µM; [DOC], 3.2 µM. Results are presented as the mean, error bars represent SEM and n=3. Inhibition was compared for each concentration using one-way analysis of variance followed by Tukey comparison test (***, P<0.001).

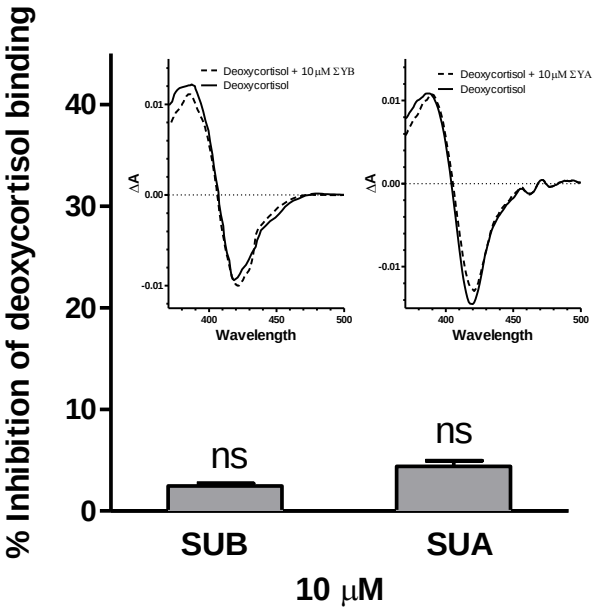

Fig. 4.12: Inhibition of deoxycortisol binding in ovine adrenal mitochondria in the presence of 10 µM SUB or SUA. [P450 mitochondria], 0.80 µM; [Deoxycortisol], 3.2 µM. Results are presented as the mean, error bars represent SEM and n=3. Inhibition was compared for each concentration using one-way analysis of variance followed by Tukey comparison test (ns, non-significant).

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We previously showed (Prevoo et al. 2008), using a mixture of triterpenoids at higher concentrations, that PROG binding was inhibited, while lower concentrations had no significant effect on PREG binding. It is possible that SUC and SUD, also present in the mixture, may have contributed to the inhibition previously observed. Nevertheless, the inhibition of substrate binding to P450 enzymes observed in the presence of the sutherlandiosides reflects the inhibition of substrate binding observed in the presence of *S. frutescens*.

Conversion assays in COS-1 cells

We investigated the influence of SUB on the catalytic activity of CYP17A1 and 3β-HSD2, branch point enzymes in adrenal steroidogenesis, towards PREG, and on that of CYP17A1 and CYP21A2 towards PROG. While the conversion of both PREG and PROG by CYP17A1 was inhibited significantly ($P<0.01$) in the presence of 30 µM SUB (Fig. 4.13A and B), it was only the catalytic activity towards PROG that was significantly inhibited at the lower concentration ($P<0.05$). The catalytic activity of CYP21A2 towards PROG (Fig. 4.13C) was not influenced by SUB. In contrast, 3β-HSD2 was inhibited significantly ($P<0.01$) at both concentrations of SUB (Fig. 4.13D). In addition, the catalytic activity of CYP11B1 towards DOC (Fig 4.13E) and deoxycortisol (Fig. 4.13F) was not influenced by the presence of SUB, whereas DOC conversion by CYP11B2 (Fig. 4.13G) was significantly ($P<0.05$) inhibited.

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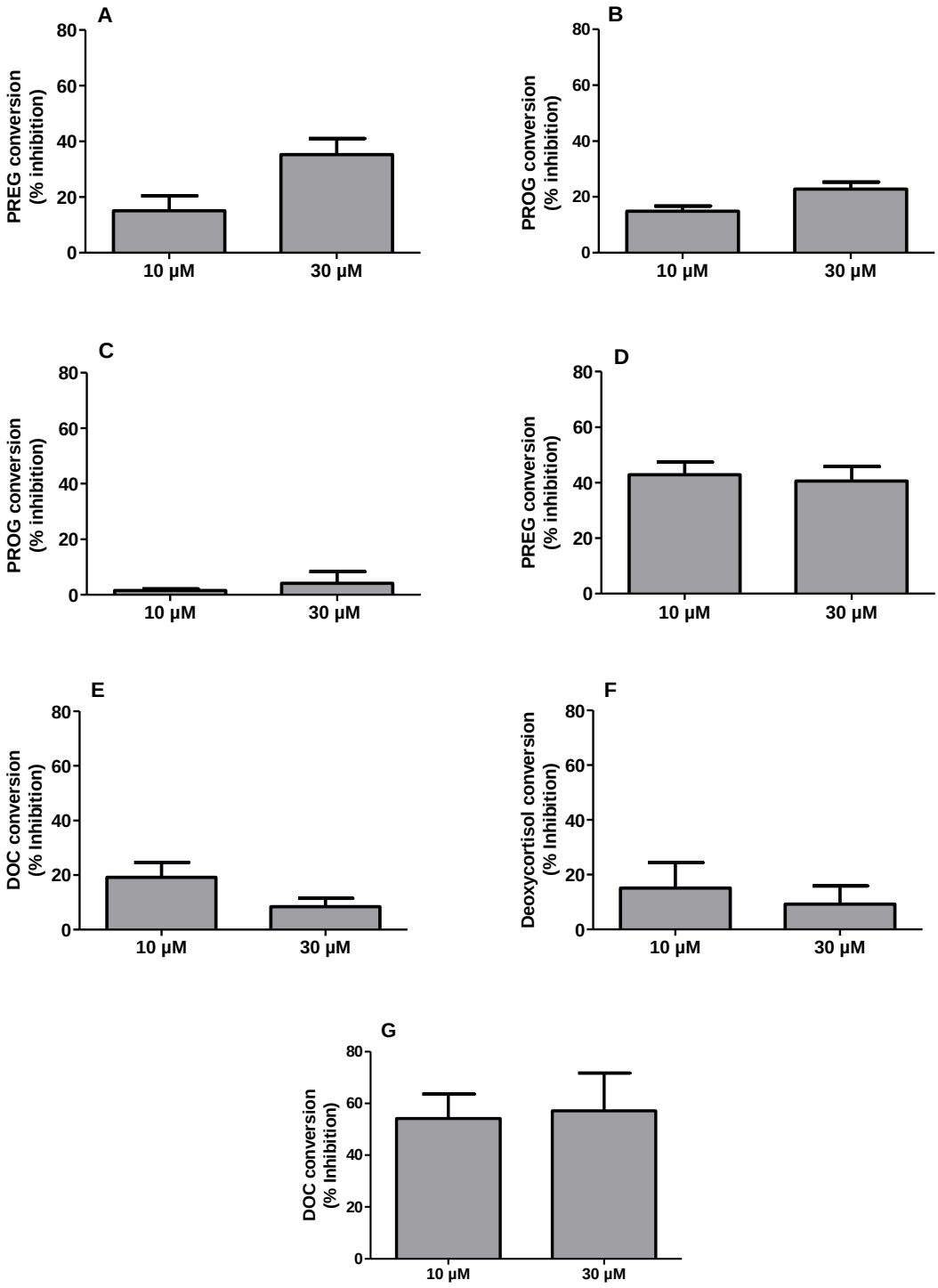

Fig. 4.13: Inhibition of substrate (1 µM) conversion by SUB in transiently transfected COS-1 cells. Percentage inhibition of (A) PREG and (B) PROG conversion by CYP17A1; (C) PROG conversion by CYP21A2 and (D) PREG conversion by 3β-HSD2; (E) DOC and (F) deoxycortisol conversion by CYP11B1 and (G) DOC conversion by CYP11B2 in the presence of 10 and 30 µM SUB.

The data show that the enzymes most sensitive to SUB, at the lower and higher concentrations, were CYP11B2 and 3β-HSD2 with a 40-50% inhibition being observed. It was only at the

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higher concentration of SUB that the catalytic activity of CYP17A1 was affected with the inhibition of PREG's conversion being marginally greater than that of PROG.

Steroid production in H295R cells is influenced by SUB

In the experiments described above SUB was shown to inhibit the catalytic activities of branch point steroidogenic enzymes, CYP17A1 and 3β-HSD2, and as such may thus affect steroidogenesis. Due to limited availability, the effect of SUB was not assayed under forskolin or AngII stimulated conditions. However, the effect of 30 µM SUB on adrenal steroidogenesis was compared to the effect of a mixture of SU compounds at the same concentration in the adrenal H295R cell model.

The effects of SUB on steroid hormone production was first assayed under basal conditions to determine whether the compounds could modulate the steroid flux due to their inhibitory effects on the catalytic activity of CYP17A1 and 3β-HSD2. Although the reduction of total steroid production was not significant at both concentrations, steroid production appeared to decrease by ± 20% in the presence SUB (Table 4.8). The reduced levels of 16OH-PROG, which were significant at 30 µM, reflect the inhibition of CYP17A1 and, together with the significantly decreased A4 and 11OHA4 levels which suggest a reduced flux in the androgen pathway, indicates a potential additive effect of the inhibition of 3β-HSD2. In the presence of 30 µM SUB, A4 (± 990 to 670 nM), 11OHA4 (± 195 to 110 nM), 16OH-PROG (± 50 to 30 nM) and cortisol (930 to 685 nM) were significantly decreased, while 11-DHC was significantly increased (± 30 to 65 nM). The data also showed that, at the higher concentration, SUB resulted in a ± 20% and 40% decrease in the steroids in the glucocorticoid and androgen pathways, respectively. Interestingly the production of 11-DHC increased significantly indicating increased inactivation of CORT metabolism.

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Table 4.8: Steroid production in H295R cells in the absence and presence of SUB after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	Basal	10 µM SUB		30 µM SUB	
	% of Total ± SEM	% of Total ± SEM		% of Total ± SEM	
PREG	ND	ND	ns	ND	ns
PROG	0.00 ± 0.00	0.05 ± 0.00	ns	0.05 ± 0.00	ns
DOC	0.15 ± 0.00	0.20 ± 0.00	ns	0.30 ± 0.00	ns
CORT	6.30 ± 0.50	6.85 ± 1.15	ns	7.40 ± 1.55	ns
18OH-CORT	0.35 ± 0.10	0.45 ± 0.1	ns	0.50 ± 0.15	ns
ALDO	0.05 ± 0.05	0.05 ± 0.05	ns	0.15 ± 0.15	ns
11-DHC	0.70 ± 0.05	1.40 ± 0.30	ns	2.05 ± 0.55	↑ P<0.05
17OH-PREG	0.00 ± 0.00	0.00 ± 0.00	ns	0.00 ± 0.00	ns
17OH-PROG	1.15 ± 0.05	1.10 ± 0.05	ns	1.15 ± 0.05	ns
16OH-PROG	1.20 ± 0.00	1.10 ± 0.05	ns	0.90 ± 0.1	↓ P<0.05
Deoxycortisol	35.80 ± 1.75	39.30 ± 1.5	ns	40.60 ± 1.50	ns
Cortisol	23.10 ± 0.75	22.40 ± 0.4	ns	21.35 ± 0.80	↓ P<0.05
Cortisone	0.20 ± 0.05	0.15 ± 0.05	ns	0.15 ± 0.00	ns
DHEA	ND	ND		ND	
DHEAS	0.25 ± 0.00	0.14 ± 0.05	ns	0.15 ± 0.05	ns
A4	24.60 ± 0.50	21.1 ± 0.5	↓ P<0.05	21.00 ± 1.00	↓ P<0.05
11OHA4	4.85 ± 0.50	4.25 ± 0.20	↓ P<0.01	3.40 ± 0.15	↓ P<0.001
Testosterone	1.30 ± 0.05	1.05 ± 0.15	ns	0.90 ± 0.20	ns
Total steroid (nM)	4020.00 ± 90.00	3200.00 ± 650	ns	3300 ± 1235	ns

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These results prompted the investigation into the effects of the triterpenoid mixture which contained SUA as well as SUC and SUD. Two concentrations were assayed in which the sutherlandiosides, SUA, SUB, SUC and SUD were present as follows: at 2.38, 3.06, 1.90 and 2.66 µM in the 10 µM SU mixture and 7.1, 9.2, 5.7, and 8 µM in the 30 µM mixture, respectively. It is possible that the triterpenoids may act either synergistically or competitively as our data showed that SUA and SUB interact differently with the P450 enzymes in terms of binding (Figs 4.10 -4.12). Table 4.9 shows that the steroid production in H295R cells when exposed to the SU mixture at either concentration, was not reduced significantly, with only cortisol (± 930 to 640 nM) concentrations decreasing significantly in the presence of both 10 and 30 µM SU mixture. The data show that the triterpenoids were not able to elicit the same effects as SUB suggesting that the compounds do not act synergistically.

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Table 4.9: Steroids produced by H295R cells in the absence and presence of SU mixture (SU) after 48 hours. The data represents the average of two independent experiments conducted in triplicate (n=6).

Steroid metabolite	Basal	10 µM SU		30 µM SU	
	% of Total ± SEM	% of Total ± SEM		% of Total ± SEM	
PREG	ND	ND	ns	ND	ns
PROG	0.02 ± 0.00	0.03 ± 0.01	ns	0.04 ± 0.01	ns
DOC	0.17 ± 0.02	0.24 ± 0.03	ns	0.27 ± 0.02	ns
CORT	6.31 ± 0.60	7.23 ± 1.36	ns	7.38 ± 1.54	ns
18OH-CORT	0.33 ± 0.11	0.44 ± 0.12	ns	0.49 ± 0.13	ns
ALDO	0.07 ± 0.04	0.05 ± 0.03	ns	0.14 ± 0.14	ns
11-DHC	0.69 ± 0.03	1.94 ± 0.90	ns	2.04 ± 0.57	ns
17OH-PREG	0.00 ± 0.00	0.00 ± 0.00	ns	0.00 ± 0.00	ns
17OH-PROG	1.13 ± 0.06	1.07 ± 0.08	ns	1.16 ± 0.06	ns
16OH-PROG	1.19 ± 0.02	1.10 ± 0.06	ns	0.89 ± 0.08	ns
Deoxycortisol	35.79 ± 1.76	38.10 ± 1.35	ns	40.59 ± 1.51	ns
Cortisol	23.12 ± 0.75	21.78 ± 0.91	↓ P<0.05	21.35 ± 0.79	↓ P<0.05
Cortisone	0.18 ± 0.03	0.16 ± 0.04	ns	0.13 ± 0.02	ns
DHEA	ND	ND	ns	ND	ns
DHEAS	0.23 ± 0.02	0.17 ± 0.03	ns	0.16 ± 0.03	ns
A4	24.61 ± 0.40	21.66 ± 1.97	ns	21.03 ± 1.07	ns
11OH-A4	4.84 ± 0.30	4.13 ± 0.38	ns	3.41 ± 0.15	ns
Testosterone	1.31 ± 0.06	1.06 ± 0.17	ns	0.91 ± 0.18	ns
Total steroid (nM)	4130 ± 132	2970 ± 1000.00	ns	3200 ± 900.00	ns

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The effect of the *S. frutescens* extract and SUB on downstream steroidogenic effects, specifically GR and MR mediated gene transcription.

The ability of the extract and SUB to influence adrenal glucocorticoid and mineralocorticoid production, as shown in the previous results, may suggest that the extract and SUB could be used as a herbal remedy in aiding the treatment of inflammation, hypertension and/or chronic disease. These results prompted the investigation into the methanolic extract and SUB eliciting genomic effects mediated via the GR and MR.

***S. frutescens* and SUB are GR agonists for transrepression but not transactivation.**

The GR and MR are ligand-dependent transcription factors that exhibit significant similarity in both their structures and mechanism of action. Like all steroid receptors, the GR and MR regulate gene expression by activating (transactivation) or repressing (transrepression) transcription of target genes (Nicolaides et al., 2010; Nixon et al., 2013). Transactivation occurs when the ligand-activated GR or MR binds to GREs or MREs in the promoters of specific genes, while transrepression occurs via protein-protein interactions with other transcription factors such as NF-κB, which drives the expression of many genes involved in the inflammatory response (Silverman and Sternberg, 2012).

The relative glucocorticoid properties of the *S. frutescens* extract and SUB for transactivation of a synthetic GRE and transrepression of an IL-6 promoter-reporter gene were assayed in COS-1 cells. For transactivation, the cells transiently expressing hGR and GRE-driven rat TAT-GRE-luciferase were treated with the synthetic glucocorticoid, Dex (10 µM), extract (0.5 and 0.75 mg/mL) and SUB (10 and 30 µM). Results show that, unlike Dex, neither the extract (Fig. 4.14A) nor SUB (Fig. 4.14B) display glucocorticoid agonist activity via the GRE at either concentration tested.

To compare the relative glucocorticoid properties of the extract and SUB for transrepression, COS-1 cells were transiently transfected with the hGR expression vector and the IL-6-NF-κB luciferase promoter reporter construct. After stimulation with 10 ng/mL PMA cells were exposed to Dex (10 µM), extract (0.5 and 0.75 mg/mL) and SUB (10 and 30 µM). The results in Fig. 4.15A and 4.15B showed that Dex, *S. frutescens* extract and SUB significantly repressed the IL-6 promoter ($P<0.001$) with Dex, as expected, displaying the greatest transrepression. In addition, the results show that the extract elicited a greater effect on the transrepression of gene transcription by the GR than SUB.

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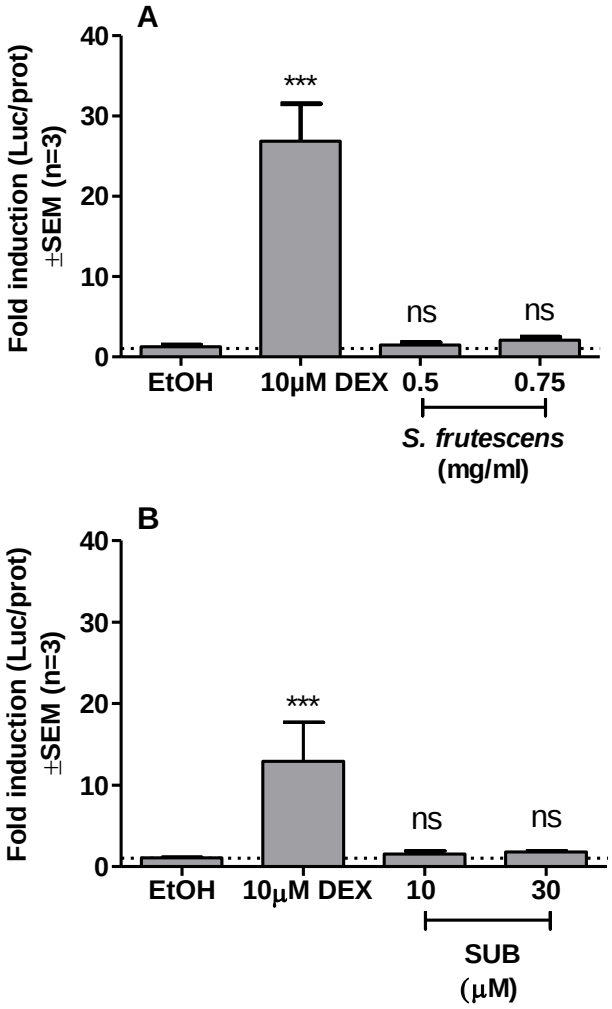

Fig. 4.14: *S. frutescens* and SUB exhibit no GR agonist activity for transactivation. Induction was assayed after 24 hours in COS-1 cells transiently transfected with pRShGR and pTAT-GRE-E1b-luc reporter constructs exposed to vehicle (EtOH) and DEX (10 µM), (A) *S. frutescens* extract (0.5 and 0.75 mg/mL) and (B) SUB (10 and 30 µM). Results are shown as fold induction relative to EtOH set as 1 and results are representative of two independent experiments performed in triplicate. One-way ANOVA with Dunnett's post-test was used for statistical significance (***, P<0.001; ns, non-significant).

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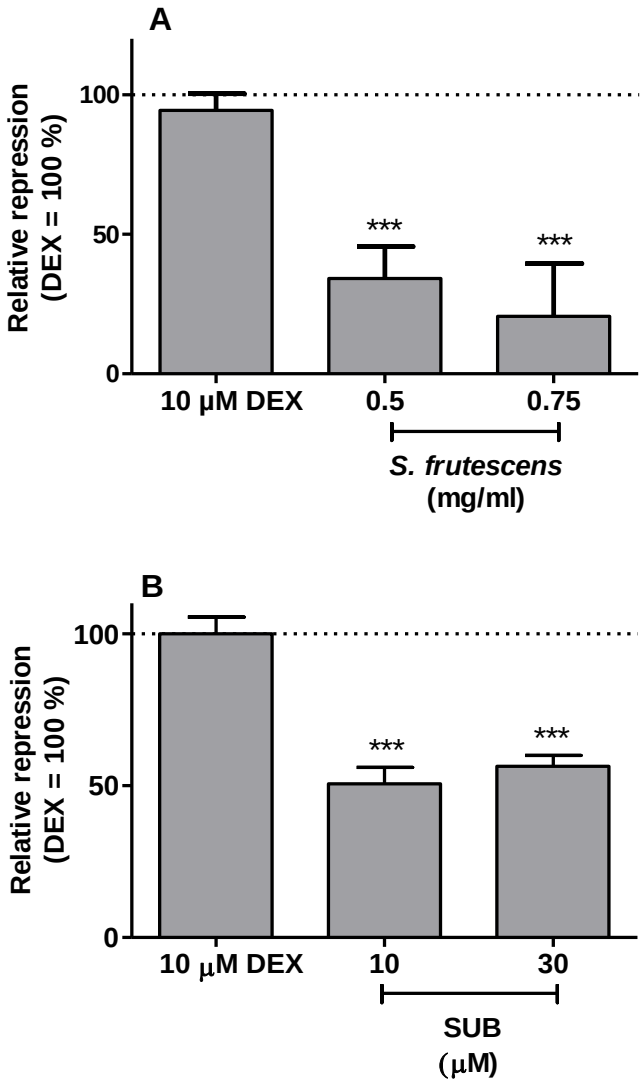

Fig. 4.15: *S. frutescens* and SUB exhibit GR agonist activity for transrepression. COS-1 cells transiently expressing the pRShGR and IL-6-NFκB-luc promoter-reporter construct were stimulated with PMA (10 ng/mL) and exposed to DEX (10 µM) and (A) *S. frutescens* extract (0.5 and 0.75 mg/mL) and (B) SUB (10 and 30 µM) for 24 hours. Results are shown as the % repression of the extract and SUB expressed as a % relative to the DEX response set as 100%. Results are representative of two independent experiments each performed in triplicate. One-way ANOVA with Dunnett's post-test was used for statistical significance (***, P<0.001; ns, non-significant).

S. frutescens and SUB exhibit MR antagonist activity for transactivation.

We subsequently assayed the putative MR antagonist activity of the extract and SUB in CHO cells stably expressing the rat MR, 11β-HSD2 and a pTAT3-gLuc reporter gene (Morita et al., 1996). Cells were incubated with ALDO (10 nM), extract (0.5 and 0.75 mg/mL) and SUB (10 and 30 µM) or with 10 nM ALDO in the presence of extract (0.5 and 0.75 mg/mL), SUB (10 and 30 µM), or SPL (1 µM). The results in Fig. 4.16A and B show that neither the extract nor SUB display significant agonist activity for the MR at the concentrations assayed. However,

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the results in Fig. 4.17A and B show that like SPL, the well-known MR antagonist, the effects of ALDO via the MR can be antagonized by both the extract (0.5 and 0.75 mg/mL) and SUB (10 and 30 µM). Less antagonism is observed in the case of SUB ($P<0.05$) (Fig. 4.17B) than with the *S. frutescens* extract (Fig. 4.17A), which antagonized the effect of ALDO, comparable to that achieved by SPL ($P<0.001$).

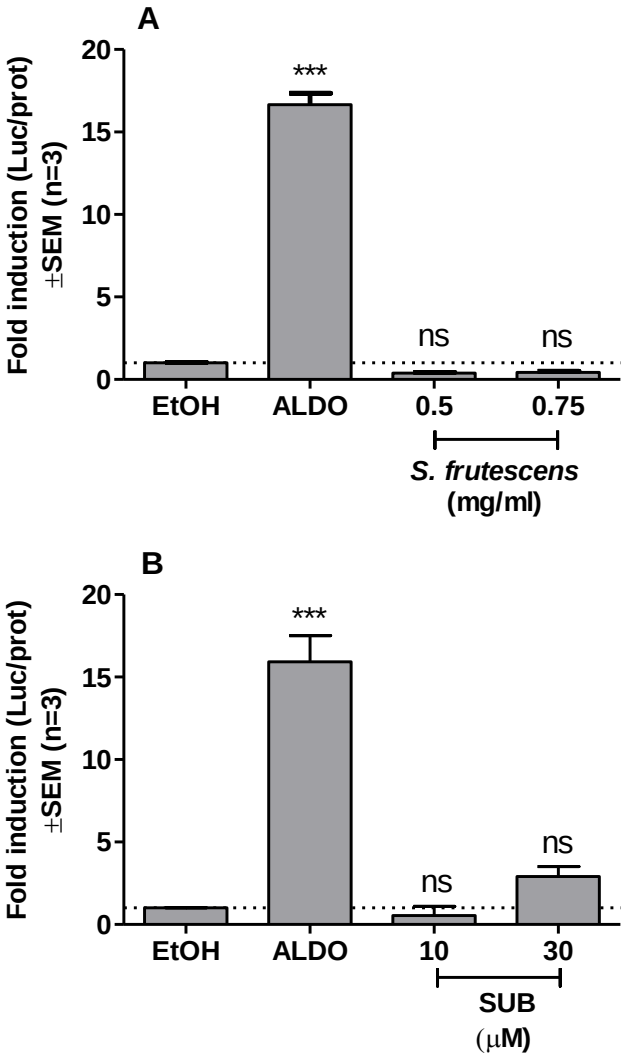

Fig. 4.16: *S. frutescens* and SUB exhibit no MR agonist activity for transactivation. CHO-11BHSD2 cells stably expressing rat MR and the pTAT3-gLuc reporter gene were incubated with vehicle (EtOH) and ALDO (10 nM) and (A) *S. frutescens* extract (0.5 and 0.75 mg/mL) and (B) SUB (10 and 30 µM) for 24 hours. Results shown are representative of two independent experiments performed in triplicate. Fold induction relative to EtOH set as 1 is plotted. One-way ANOVA with Dunnett's pos-ttest was used for statistical significance (***, $P<0.001$; ns, non-significant).

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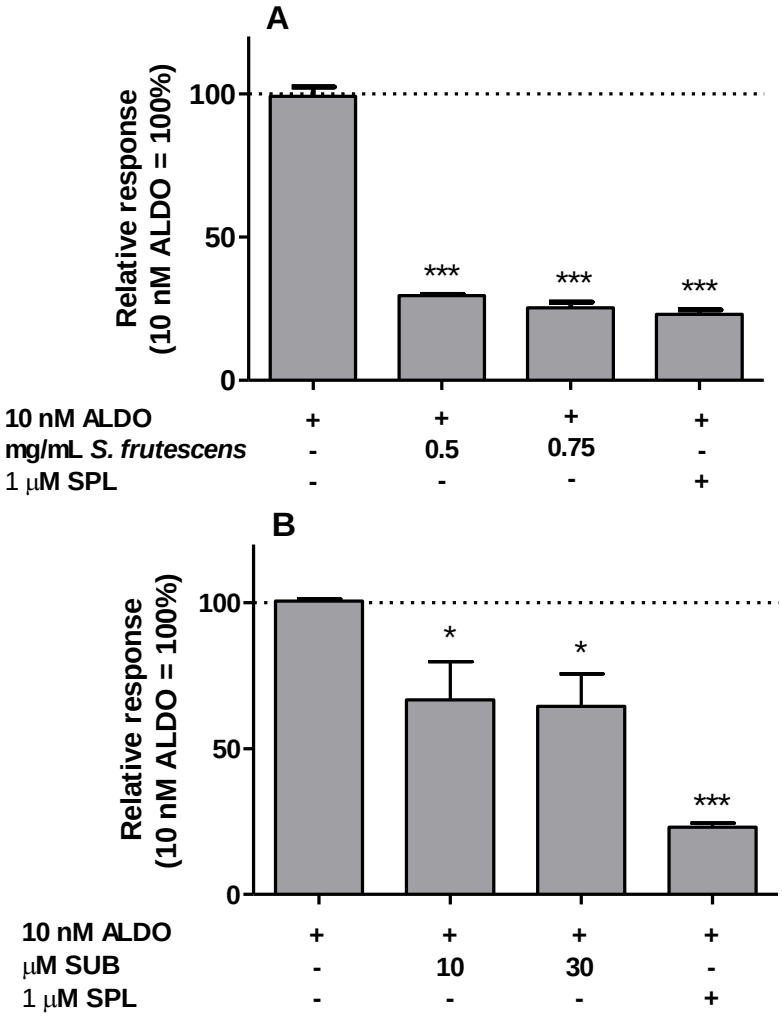

Fig. 4.17: *S. frutescens* and SUB exhibit MR antagonist activity for transactivation. CHO-11BHSD2 cells stably expressing rat MR and pTAT3-gLuc reporter gene were incubated with ALDO (10 nM), set at 100%, or (A) ALDO in the presence of *S. frutescens* extract (0.5 and 0.75 mg/mL) or SPL (1 µM), and (B) ALDO in the presence of SUB (10 and 30 µM) or SPL (1 µM) for 24 hours. Results shown are representative of two independent experiments performed in triplicate. One-way ANOVA with Dunnett's post-test was used for statistical significance (***, P<0.001; **, P<0.01).

Since the MR is also susceptible to activation by deoxycortisol and DOC, we investigated whether the extract would have a similar antagonizing effect in the presence of these glucocorticoids. Cortisol and CORT were not considered in this investigation as CHO cells stably express 11β-HSD2 which converts cortisol and CORT to their inactive metabolites cortisone and 11-DHC, respectively. Figure 4.18 and 4.19 show that the *S. frutescens* extract antagonized the effects of both DOC (P<0.001) and deoxycortisol (P<0.001) via the MR and

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the antagonist activity observed for the extract is comparable to that of SPL in both cases. The effect of SUB was not assessed due to limited availability of SUB.

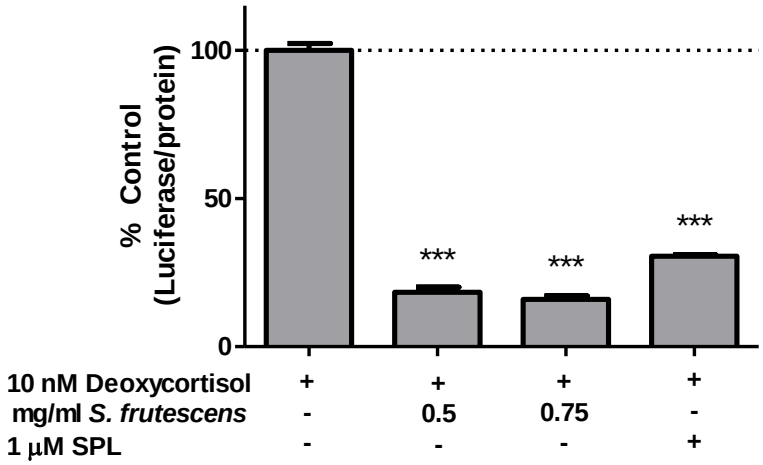

Fig. 4.18: *S. frutescens* MR antagonist activity for transactivation in the presence of deoxycortisol. CHO-11BHSD2 cells stably expressing rat MR and the pTAT3-gLuc reporter gene were incubated with deoxycortisol (10 nM) set at 100% or deoxycortisol in the presence of *S. frutescens* extract (0.5 and 0.75 mg/mL) or SPL (1 µM) for 24 hours. Results are representative of two independent experiments performed in triplicate (±SEM). One-way ANOVA with Dunnett's post-test was used for statistical significance (***, P< 0.001).

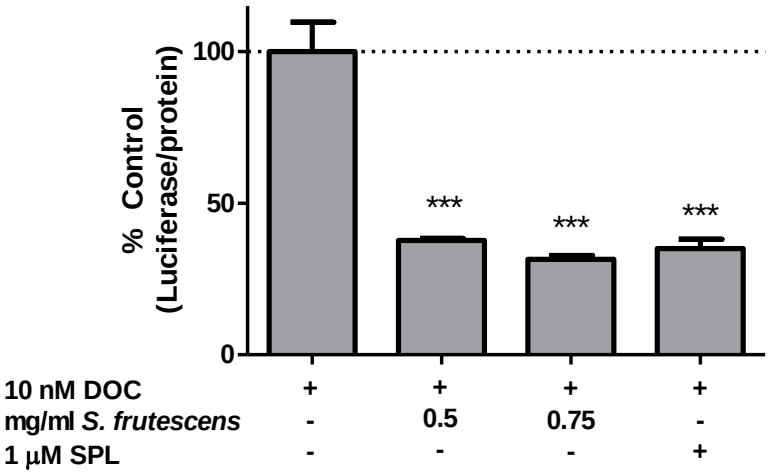

Fig. 4.19: *S. frutescens* MR antagonist activity for transactivation in the presence of DOC. CHO-11BHSD2 cells stably expressing rat MR and the pTAT3-gLuc reporter gene were incubated with DOC (10 nM) set at 100% or DOC in the presence of *S. frutescens* extract (0.5 and 0.75 mg/mL) or SPL (1 µM) for 24 hours. Results are representative of two independent experiments performed in triplicate (±SEM). One-way ANOVA with Dunnett's post-test was used for statistical significance (***, P< 0.001).

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Cell viability assays

The cell viability of the COS-1, H295R and stably transfected CHO cells were assessed using the MTT assay. The MTT assay is based on the difference in absorbance observed in the absence and presence of the test compound as a measure of mitochondrial succinate dehydrogenase activity. MTT is reduced from its yellow form to a dark purple formazan product, which is dissolved using an organic solvent and the solution is measured spectrophotometrically. This reaction can only occur in cells that are metabolically active and is therefore a measure of cell viability. Our results show that there was no significant difference between the control reactions and the reactions in the presence of the extract or SUB (all cell lines) (Fig 4.20A-C) or GABA (H295R cells only) (Fig 4.20B).

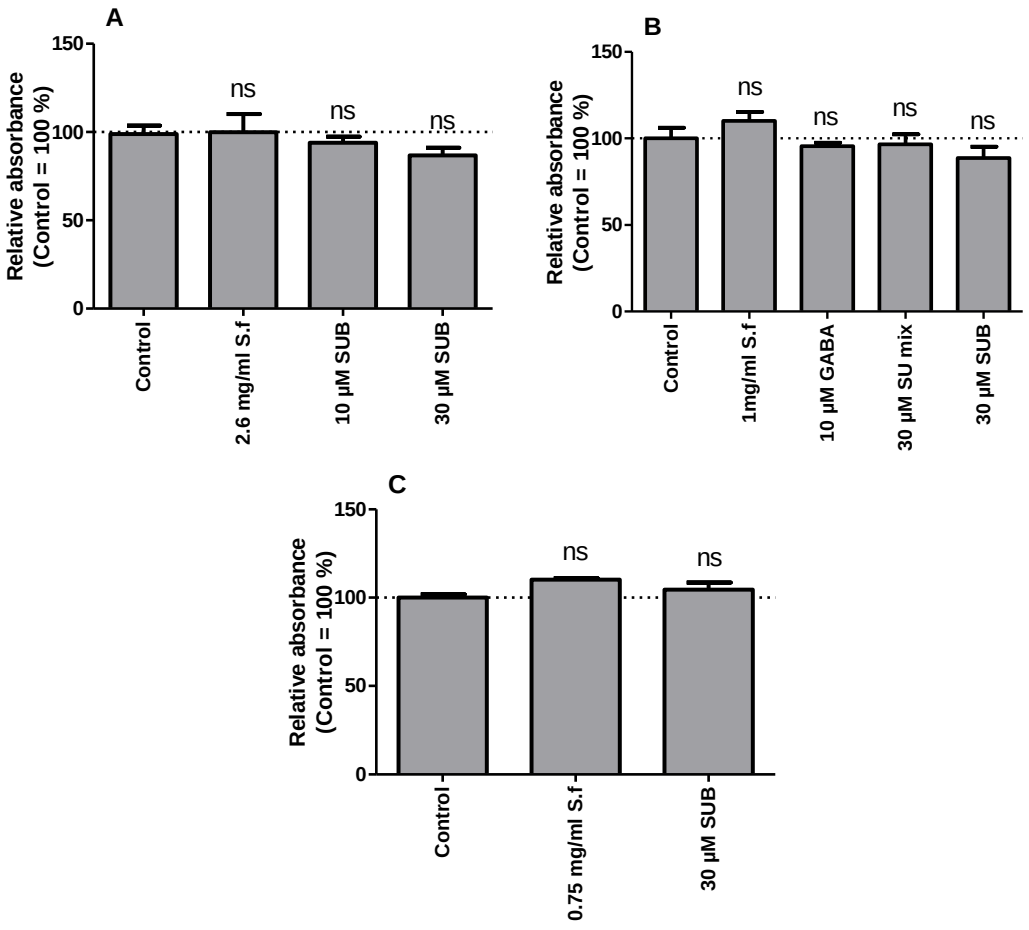

Fig. 4.20: *S. frutescens* extracts (S.f), GABA, SU mixtures or SUB did not affect cell viability. The cell concentrations used were (A) $1x10^5$ cells/mL for COS-1 cells, (B) $4x10^5$ cells/mL for H295R cells and (C) $2.5x10^4$ cells/mL for CHO cells. The concentrations of extract and compounds added to the cells are indicated on the graphs. A one-way ANOVA, followed by a Dunnett's comparison test was used to determine significant differences (ns, non-significant).

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Discussion

As disorders such as stress, inflammation and anxiety for which *S. frutescens* commonly serves as a tonic are generally associated with adrenal dysfunction, we assessed the influence of *S. frutescens* and SUB on adrenal steroidogenesis in order to establish a potential link. Data show that the methanolic extract of *S. frutescens* as well as SUB influence adrenal steroid production through influencing the catalytic activity of specific P450 enzymes as well as steroid production in the H295R adrenal cell model affecting steroidogenic pathways. Furthermore, potential downstream effects were investigated in which the extract and SUB were shown to influence the transcriptional activities of the GR and MR. Both the extract and SUB repressed NF-κB driven transcription via the GR without influencing transactivational activities and were able to antagonize the effects of ALDO via the MR.

Although the inhibitory effect of *S. frutescens* on CYP17A1 and CYP21A2 was previously investigated (Prevoo et al., 2008, 2004), an aqueous extract was utilized whereas a methanolic extract was used in this study. Due to the differences in the extraction methods employed, we could not assume that the methanol extract would have similar effects on the catalytic activities of the enzymes. Our study confirmed the inhibitory effect of *S. frutescens* on steroid conversion catalysed by CYP17A1 and CYP21A2, as was also shown with the aqueous *S. frutescens* extract, although there were differences in the results obtained. Although the inhibition of PROG conversion by both CYP17A1 and CYP21A2 was confirmed, the methanolic extract appeared to have no inhibitory effect on PREG conversion by CYP17A1. As expected, no A4 was detected when the conversion of PROG by CYP17A1 was investigated, which can be attributed to species specific differences in the CYP17A1 lyase activity. Prevoo et al. utilized ovine adrenal microsomes and recombinant ovine CYP17A1, whereas we utilized baboon CYP17A1. Hough et al. has shown that numerous ovine CYP17A1 isoforms, which differ with regard to lyase activity, exist naturally and therefore influence the conversion of PREG and PROG as well as downstream cortisol production to different degrees (Hough et al., 2015, 2012).

In addition to the inhibition of the catalytic activity of CYP17A1 and CYP21A2, this study also found that 3β-HSD2 was partially inhibited by the presence of the methanolic extract (Fig. 4.1). Although the conversion of PREG and DHEA catalysed by 3β-HSD2 was not inhibited, the conversion of 17OH-PREG to 17OH-PROG was. The impaired production of 17OH-PROG by 3β-HSD2 in Angora goats has been linked to hypocortisolism (Goosen et al., 2010) and thus the inhibition of 17OH-PROG formation in the presence of the methanolic extract may also

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indicate that adrenal cortisol output may be reduced by the extract. Taken together with the observed inhibition of CYP17A1 and CYP21A2, the results suggest that the extract may inhibit the production of glucocorticoids and mineralocorticoids. The collective inhibitory effect of both 3β-HSD2 and CYP17A1 would be associated with reduced cortisol production, whereas the collective inhibitory effect of both 3β-HSD2 and CYP21A2 would also be associated with reduced mineralocorticoid production.

The final step in adrenal glucocorticoid production is the conversion of DOC and deoxycortisol to CORT and cortisol, respectively. DOC plays an integral role in glucocorticoid and mineralocorticoid production as this steroid metabolite can either be converted to CORT (a glucocorticoid) by CYP11B1 or ALDO (the major mineralocorticoid) by CYP11B2. In addition to acting like a glucocorticoid in many functions such as the stress response, DOC has also been shown to act as a significant mineralocorticoid in downstream signalling events (Vinson, 2011). DOC and deoxycortisol have also been shown to play a significant role in hypertension and via the MR (Sugimoto et al., 2016). The inhibitory effect of the methanol extract on cortisol and CORT production by CYP11B1 may thus have substantial implications in disorders concerning both glucocorticoid and mineralocorticoid abnormalities.

The results shown in Table 4.1 show that glucocorticoid production in H295R cells was affected to the greatest degree in the presence of the methanol extract as the most significant decreases in steroid concentration were observed for CORT (2.7-fold), deoxycortisol (1.3-fold) and cortisol (1.9-fold), while DOC concentrations were increased (1.6-fold). However, when taking into account the steroid shunt in the mineralocorticoid, glucocorticoid and androgen pathway, the results show that the greatest inhibitory effect was observed for mineralocorticoid production with the extract decreasing the total mineralocorticoids present by 1.7-fold together with glucocorticoid production which decreased 1.5-fold (± 2470 nM to 1670 nM), while androgen production, which was the least affected, decreased 1.2-fold (1245 nM to 1055 nM). The results also confirmed the aforementioned findings which showed that DOC production by CYP21A2 and DOC conversion by CYP11B1 were inhibited by the extract as the DOC levels in the presence of *S. frutescens* was ± 2.4-fold higher than that of basal DOC levels. Furthermore, SULT2A1 appears to be stimulated by the presence of the extract as DHEAS production was shown to be ± 2.5-fold greater than levels at basal production.

The inhibition of CYP21A2 and CYP11B2 are effects that reflect data obtained in the catalytic assays conducted in COS-1 cells (Fig. 4.3 and 4.5) in which PROG and DOC levels were

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increased. In addition, the increased 17OH-PROG and the decreased deoxycortisol and cortisol in the H295R cell assay also indicated the inhibition of CYP21A2 and CYP11B1, respectively. It should be noted that the decreased 16OH-PROG levels suggest the inhibition of CYP17A1 which was also shown in COS-1 cells in which the 17-hydroxylation of PROG was inhibited significantly (Fig. 4.2).

The effect of *S. frutescens* on adrenal steroid production during chronic stress was mimicked by adding the extract to forskolin stimulated cells. Forskolin mimics ACTH stimulation in H295R cells and as expected increased glucocorticoid (2.4-fold), mineralocorticoid (6.6-fold) and androgen production (1.7-fold) in these cells (Table 4.2). Overall steroid production was increased 2.7-fold with the results obtained in this study being similar to those previously reported (Mangelis et al., 2016; Schloms et al., 2012; Xing et al., 2010). In the presence of the methanolic extract, overall steroid production decreased when compared to forskolin stimulation only (1.7-fold), however, the overall steroid production in the presence of the extract after forskolin stimulation remained 1.6-fold greater than steroid production under basal conditions.

The greatest inhibitory effect observed under forskolin stimulatory conditions in the presence of the extract was the decrease in mineralocorticoid production (2.2-fold), followed by decreased glucocorticoid (1.6-fold) and androgen production (1.5-fold). As in the previous experiment, the presence of the extract appeared to favour the sulfonation of DHEA, even under forskolin stimulated conditions, as DHEAS concentrations increased 2.8-fold. In addition, basal testosterone levels were decreased 1.2-fold by the extract under stimulated conditions and this apparent decrease could point to a potential inhibitory effect elicited by the extract on 17β-HSD with this enzyme belonging to the family of oxidoreductases, in the same steroid family as 3β-HSD2. The extract was shown to partially inhibit the catalytic activity of 3β-HSD2 in the conversion assays (Fig. 4.1). It is due to this theory that the MTT assay was employed for determining cell viability during this study as the potential inhibitory effect of the extract on the catalytic activity of 17β-HSD may have resulted in ambiguous results. In the scope of this study, the measure of reductive capacity of the mitochondria, as measured by the MTT assay, was considered be a superior indicator of cell viability compared to steroid conversion by 17β-HSD. However, it has been shown that the activity of 17β-HSD towards A4 was not inhibited significantly with approximately 90% of the A4 being converted to testosterone (Sergeant, 2009).

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Also belonging to the hydroxysteroid dehydrogenase family are the 11-βHSD isozymes, both of which are expressed in the adrenal at low levels (Rege et al., 2013), accounting for the production of cortisone and 11-DHC, albeit at low levels. In the presence of forskolin both cortisone and 11-DHC levels increased (1.5-fold and 2.1–fold, respectively), with only 11-DHC significantly increased by the extract ($P<0.001$). While AngII did not increase 11-DHC levels significantly, even though precursor CORT levels were elevated, the addition of the extract to the cells had no effect on the inactivation of cortisol or CORT. However, considering the active:inactive glucocorticoid ratios, the addition of the extract to basal and to both forskolin- and AngII-stimulated H295R cells resulted in decreased cortisol:cortisone and CORT:11-DHC ratios. These data suggest an interaction between the extract, and/or compounds within the extract, and 11-βHSD2 which could likely result in decreased circulatory active glucocorticoids, further reducing glucocorticoid-elicited effects at target tissues which would, in turn, affect the stress or inflammatory response.

It is possible that more marked effects on 11-βHSD2 may have been observed had the stimulation by AngII been more significant than that reflected by the significant increase in CORT alone together with the marginal increases in certain metabolites and in total steroid production. Previous studies have shown that the addition of AngII to H295R cells resulted in significant stimulation of adrenal steroid production, specifically the mineralocorticoids and mineralocorticoid precursors (Mangelis et al., 2016; Yarimizu et al., 2015). The steroid profile in these studies differ from the results depicted in Table 4.5 and the differences could be attributed to the AngII concentrations. In the studies by Yarimisu et al. (2015) and Mangelis et al. (2016) 100 nM AngII was used, whereas our study used a concentration closer to that used in the study by Lichtenauer et al. (2012) (20 nM) (Lichtenauer et al., 2012; Mangelis et al., 2016; Yarimizu et al., 2015). The latter study also showed that a greater mineralocorticoid response was observed after forskolin stimulation, rather than after AngII stimulation, a result similar to that which was obtained in this study. Forskolin increased mineralocorticoid production in the H295R cells 6.6-fold, whereas stimulation by AngII resulted in a 1.5-fold increase. The only steroid metabolite that was significantly increased by AngII stimulation was CORT (1.8-fold). The increase in CORT production is significant with regard to mineralocorticoid production with a 1.5-fold increase in metabolites in the mineralocorticoid pathway after AngII stimulation, whereas glucocorticoid production remained unchanged (basal, 2965 nM and AngII stimulation, 3040 nM). Basal androgen metabolite (C_{19} steroids) levels did not change significantly in the presence of AngII (1307 nM vs 1335 nM). Previous

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studies have shown that AngII stimulates the expression of CYP11B2 (Razak et al., 2015) and 3β-HSD2 mRNA in H295R cells (Ota et al., 2014), and although the increase in mineralocorticoids detected in this study after AngII stimulation may reflect this, the concentrations of AngII used in this study is approximately 10-fold less than that used in the previously reported studies. In addition, it has been reported that the expression of StAR mRNA was also upregulated after AngII stimulation (Olala et al., 2014) which would impact on cholesterol's availability for PREG production.

The therapeutic effects associated with the traditional use of *S. frutescens* could be attributed to specific compounds or compounds acting synergistically. Several compounds, such as L-canavanine, D-pinitol, and GABA have been identified in methanolic extracts of *S. frutescens*, however, these compounds have not been shown to interfere with steroidogenic P450 enzyme activity. As our previous results have shown, the methanolic extract of *S. frutescens* influenced glucocorticoid and mineralocorticoid production by altering the catalytic activities of the steroidogenic enzymes. These results prompted us to investigate the effects of the cycloartanes on steroidogenesis, with our primary focus on SUB, to determine whether the therapeutic effects associated with the use of *S. frutescens* could be attributed to the presence of these compounds. Initial investigations included SUA however, due to insufficient amount of the compound, studies could not be completed with SUA.

Our study confirmed the presence of SU A, B, C and D in the methanolic extract by LC-MS/MS and confirmed SU A and B to be the major triterpenoids in *S. frutescens* extracts. Although the SU compounds have similar cycloartane glycoside structures, they do however, differ markedly in their aglycone backbone specifically in terms of hydroxyl and ketone moieties at C1, C3, C7 and C11. In addition, SUA has a C9/C11 double bond and an epoxy spanning C7/C10 over a 7-carbon ring structure while SUB, C and D have a methylene bridge between C9 and C10 (Avula et al., 2010; Fu et al., 2008). These structural differences permit these compounds to retain defined three dimensional structures which may contribute to different bioactive properties. In this study we turned our attention to SUB as the compound was also present at concentrations marginally higher than the other SU compounds in our extract. While extraction methods would contribute to the SU yields, studies have shown that secondary metabolites present in *S. frutescens* vary according to geographical location of plants, harvesting seasons and processing methods (Albrecht et al., 2012; Shaik et al., 2011). SUB specifically, has been shown to vary 0 to 1 % (of dry mass) of *S. frutescens* plants grown in different geographical locations (Albrecht et al., 2012). Plants grown *in vitro* showed different secondary metabolite

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profiles which may be caused by the pH differences and soil that field specimens were cultivated in (Shaik et al., 2010). Our results are in agreement with the expected range and the SUB yield constituted 0.3% of the extracted dry mass of *S. frutescens*.

Since the SUs have structures similar to those of steroid hormones, it is plausible to assume that these compounds may interact with the adrenal P450 enzymes catalysing hormone biosynthesis. The P450 enzyme family, comprising of hepatic and steroidal P450 enzymes, are structurally conserved and studies suggest that herbal remedies which interact with hepatic P450 enzymes could interact with the steroidal P450 enzymes due to the structural homology between these enzymes. These interactions are well documented and it has been demonstrated that adverse events associated with herb-drug interactions may be attributed to the inhibition of hepatic P450 enzymes by bioactive compounds (Mrozikiewicz et al., 2010; Shi and Klotz, 2012; Zhou et al., 2003). Triterpenoid compounds in plant extracts have been linked to pharmacological effects which include the lowering of serum cholesterol levels as well as anti-inflammatory, cardiovascular and hypoglycemic effects (Desai et al., 2009; Han and Bakovic, 2015; Lockyer et al., 2012; Wu et al., 2011). The mechanisms through which these compounds elicit the aforementioned biological effects remain mostly unknown. While we have reported the modulation of adrenal P450 enzymes (Prevoo et al., 2004, 2008), a more recent study reported on *S. frutescens* extracts modulating P450 enzymes in human liver microsomal preparations, inhibiting the catalytic activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9 and CYP2C19 towards their known substrates in a dose dependent manner (Fasinu et al., 2013). An earlier study reported that SUB could influence drug metabolism by inhibiting CYP3A4, however no significant effect on CYP2D6 activity was reported (Madgula et al., 2008). In addition, it has been shown that the ginsenosides present in ginseng are not only metabolized by hepatic P450 enzymes (Qi et al., 2011), but that they also inhibit the aforementioned enzymes. Results have shown that the glycosyl moiety of the compounds influences bioactivity and has, as such been shown to contribute to the resistance of ginsenoside to its metabolism by CYP3A4 when compared to the metabolism of its aglycone. These data suggest that steric hindrances caused by the location of the glycosyl groups may prevent the ginsenosides from binding to the active pocket of CYP3A4 and from being metabolised (Hao et al., 2010). However, ginsenosides may bind sites other than the active site and may thus nevertheless hamper the catalytic activity of the P450 enzymes. We have shown that both SUA and SUB inhibited the binding of PROG to microsomal P450 enzymes comparable to the extract as reported by Prevoo et al., suggesting that these compounds may contribute

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significantly to the inhibitory effect elicited by the extract. The data does however show that the effect of SUA was markedly lower that the inhibitory effect of SUB. The inhibition of the binding of PROG could account for the increased PROG production observed in the presence of SUB in the H295R cells. This effect is most likely due to the inhibitory effect of SUB on CYP17A1, rather than CYP21A2, as the results show that SUB inhibits PROG conversion by CYP17A1 without affecting the catalytic activity of CYP21A2 significantly. In addition, the inhibition of DOC binding specifically by SUA and SUB may indicate that SUA and SUB have inhibitory effects on substrate binding to CYP11B2 and not CYP11B1 binding, as both CYP11B isoforms are present in adrenal mitochondria, while deoxycortisol is the major substrate for CYP11B1 and DOC is the major substrate for CYP11B 2 (Mornets et al., 1989). This effect is further mirrored in the conversion assays where SUB significantly inhibited DOC conversion by CYP11B2 while not inhibiting the catalytic activity of CYP11B1.

Although both CYP17A1 and 3β-HSD2 were inhibited by SUB when expressed in isolation, these modulatory effects are not always obvious in a complex model system endogenously expressing all the steroidogenic enzymes. Steroid conversion would, in the H295R model system, also be dependent on competition between enzymes for the same substrates, together with the availability of precursors. However, analyses indicated inhibition of CYP17A1 by SUB which would impact on the availability of substrates for the downstream conversion by 3β-HSD2 and CYP21A2.

As with SUB, mineralocorticoid production remained relatively unchanged in the presence of the SU mixture. However, the SUs decreased glucocorticoid and androgen production with the greatest inhibitory effect on androgen production (1.6-fold decrease). The only steroid metabolite that was significantly affected by the presence of the SU mixture was cortisol (1.4-fold decrease) (Table 4.9). Furthermore, the total steroid produced in the presence of the SUs was similar for both concentrations assayed and, although not significant, displayed a decreasing trend (± 1.3-fold).

Interestingly cortisol production was also decreased significantly by SUB and in the case of both the SU mixture and SUB, the cortisol:cortisone ratio increased while the CORT:11-DHC ratio decreased. Although one would expect the production of inactivated glucocorticoids to be decreased as well, it would appear that SUB affects 11β-HSD2 catalytic activity in a substrate specific manner. However, confirmation of the latter would require further investigations.

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Further analyses of steroid metabolite ratios showed that the cortisol:testosterone ratio increased, as both cortisol and testosterone levels decreased, in the presence of the SU mixture and SUB. Analyses of cortisol:testosterone ratios have enabled clinicians to establish a link with type 2 diabetes, hypertension and cardiovascular disease (Rosmond et al., 2003), as elevated ratios are also characteristic of ischemic heart disease associated with insulin resistance (Smith, 2005). In addition, decreased cortisol:testosterone ratios have also been associated with adverse behavioural changes in stressed individuals (Romero-Martínez et al., 2013). Although these effects are in conflict with the anti-inflammatory, anti-stress and anti-diabetic effects associated with the use of *S. frutescens*, the effects elicited in an animal model may be different to that observed in the cell model system as bioavailability of compounds would play a significant role in compounds reaching their target sites.

The differences in steroid metabolite production and the inhibitory effects on the catalytic activity of the steroidogenic enzymes between the SU compounds and the extracts may be attributed to the presence of other complex compounds in the extract. Several flavonoids have been identified in and isolated from *S. frutescens* extracts, with the sutherlandins constituting the greater part thereof (Avula et al., 2010; Fu et al., 2010), while rutin, vitexin and orientin have also been isolated from methanolic *S. frutescens* extracts (Sergeant, 2009). As was previously reported for flavonoids (Schloms and Swart, 2014), SUB inhibited the catalytic activity of 3β-HSD2 and CYP17A1 without affecting the activity of CYP21A2 and therefore could contribute towards the inhibitory effect of the extract towards these enzymes and steroid production in H295R cells. In addition, the SU compounds and sutherlandins could be acting synergistically in inhibiting 3β-HSD2 and CYP17A1 activity which could account for the greater inhibitory effect observed in the presence of the extract. Furthermore, rutin, vitexin and orientin were shown to inhibit the catalytic activity of CYP11B1 towards deoxycortisol to a greater degree than DOC, although rutin showed no inhibitory effect on DOC conversion by CYP11B1 (Schloms and Swart, 2014). Similar effects have been observed for flavonoids such as daidzein and genistein with earlier studies showing that these flavonoids inhibited the catalytic activity of 3β-HSD2 competitively with DHEA as substrate (Ohno et al., 2004) and to reduce cortisol production through influencing steroidogenic enzymes in H295R cells (Ohno et al., 2002).

Typically, the downstream effects of glucocorticoids and mineralocorticoids, specifically the effects associated with the inflammatory response, inflammatory mediated hypertension and

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cardio-protective effects, involve the induction of transcriptional activity mediated by the steroid receptor family, specifically the GR and MR. We thus next investigated the effect of the methanolic extract of *S. frutescens* and SUB on transcriptional activities of nuclear receptors, with specific focus on the glucocorticoid and mineralocorticoid receptors. Neither the extract nor SUB resulted in activation of transcription via the GR or MR, while both the extract and SUB efficiently repressed NF-κB-driven gene expression via the GR. The data furthermore showed that the extract and SUB antagonized the MR-mediated transactivation effects of ALDO, deoxycortisol or DOC.

Exogenous glucocorticoids acting via the GR are widely used to treat inflammatory disorders. The clinical usage of glucocorticoids is however limited, due to multiple adverse side effects associated with prolonged use which include, amongst others, mood and behavioural changes, adiposity and steroid-induced diabetes (Whitehouse, 2011). These side effects are generally reported to be due to their capacity to transactivate gene transcription (Baschant et al., 2013) while the therapeutic anti-inflammatory properties are generally considered to be due to the transrepression mechanism (Nixon et al., 2013). The latter concept was recently been called into question as it was shown that the negative side effects associated with anti-inflammatory treatments may in fact result from both transactivation as well as transrepression mechanisms of the GR (Coutinho and Chapman, 2011; Sundahl et al., 2015). However, potential anti-inflammatory treatments with fewer side effects which stimulate and ultimately modify GR-mediated gene expression profiles, are still actively being sought (Vandevyver et al., 2013). These compounds are referred to as dissociated compounds or SEGRAs (discussed in Chapter 3). One of the first SEGRAs to be described was Compound A, a non-steroidal compound isolated from *Salsola tuberculatiformis* (Swart et al., 1993), displaying no transactivation of GRE-mediated gene transcription while repressing NF-κB-driven gene expression (de Bosscher et al., 2005; Gossye et al., 2010). Collectively, our transactivation and transrepression results show that the *S. frutescens* extract and SUB can efficiently repress NF-κB-driven gene expression, without activating GRE-driven gene expression suggesting that the extract and SUB display SEGRA-like properties which may have anti-inflammatory applications. In addition to the SEGRA-like properties, it is also likely that the extract and SUB could influence the transcriptional activities of the PR and AR as these receptors share similarities in their mechanisms of action and are often able to bind to the same DNA sequence and in so doing regulate gene transcription. However, no conclusive remarks can be made regarding the effect of the extract and SUB on the PR and AR until further investigations have been conducted.

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Circulating glucocorticoids have been found to induce hypertension and although it was originally proposed that essential hypertension was mediated by sodium imbalances, recent data have suggested that the pathogenesis and maintenance of glucocorticoid-induced hypertension is mediated by the activation of the MR by glucocorticoids (Whitworth et al., 2000). Typical hypertension treatments include the administration of MR antagonists such as SPL, however research has shown a strong association between inflammation and congestive heart failure (Messaoudi et al., 2012). Although the release of pro-inflammatory cytokines are major contributing factors to the onset of inflammation-induced congestive heart failure (Dick and Epelman, 2016), glucocorticoids, the GR and the MR have also been shown to play significant roles (Fuller, 2015; Messaoudi et al., 2012). Compared to ALDO, circulating glucocorticoid concentrations are significantly higher (±1000-fold), but the binding of glucocorticoids to the MR is regulated by the presence of 11β-HSD2. As previously mentioned, 11β-HSD2 converts cortisol and CORT to their inactive forms, cortisone and 11-DHC respectively, which have no affinity for the MR (Edwards, 1988). Our results showed that neither the *S. frutescens* extract nor SUB activated MR mediated gene transcription, but instead antagonized the effects of ALDO, DOC and deoxycortisol via the MR comparable to the known MR antagonist, SPL. This result strongly suggests that the extract could be used as an anti-hypertensive treatment. The finding that *S. frutescens* also contains cardenolides, naturally occurring cardiac glycosides, also suggests a potential application as a herbal remedy in the treatment of congestive heart failure in addition to its traditional use in treating inflammation (Shaik et al., 2011).

The anti-inflammatory properties of *S. frutescens* have traditionally been regarded as a favourable effect elicited by the extract. However, recent studies have suggested that this effect may further compromise the immune system of compromised individuals as the anti-inflammatory effect may be beneficial under basal conditions, but may add to exacerbating neuro-inflammation in HIV patients (Africa and Smith, 2015). Furthermore, it is not uncommon for drug-herb interactions to affect the efficacy of drugs that require further metabolism to activate drug compounds. Although data with specific reference to the effect of *S. frutescens* in drug-herb interactions is limited, the effect of the extract on hepatic P450 enzymes (Fasinu et al., 2013) and a recent study showing reduced host reaction to conventional tuberculosis treatments after *S. frutescens* administration (Folk et al., 2016), suggest that the topic on drug-herb interactions by *S. frutescens* requires urgent attention.

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In addition to the anti-inflammatory and anti-hypertensive effects shown in this chapter, *S. frutescens* has been anecdotally linked to anti-stress and anti-anxiety and its vernacular names reflect this — musapelo (Sesotho: to turn the heart around), insiswa (Zulu: to dispel darkness) and phetola (Setswana: it changes) (van Wyk and Albrecht, 2008). In many cases the therapeutic effects of herbal medicines in the treatment of mental health disorders have been associated with the placebo effect rather than an actual medicinal effect. In the case of *S. frutescens* however, the inhibitory effect of the extract on the P450 enzymes and the resulting reduction in glucocorticoids together with the repression of NF-κB-driven gene expression, may add scientific merit to the anecdotal use of *S. frutescens* as an anti-stress and anti-anxiety therapy. Furthermore, previous studies have shown that the ingestion of *S. frutescens* resulted in reduced hippocampal GR and $GABA_A$ receptor expression (Smith and van Vuuren, 2014). As *S. frutescens* has been shown to contain GABA (Shaik et al., 2010), it is plausible that the extract may exert anxiolytic effects in a GABAergic manner. We thus next investigated the influence of GABA on steroid production in H295R cells in the next chapter.

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Chapter 5: Influence of GABA on steroidogenesis in H295R cells

Introduction

GABA, a non-protein amino acid, is found in the leaves and seed pods of many plant species and is essential for nitrogen storage. Although GABA serves as a nitrogen source in plants especially important during the germination process (Wink, 2013), in vertebrates it acts as an inhibitory neurotransmitter and has been reported to act as a non-sedative anxiolytic, mild anaesthetic and memory enhancer (Rudolph and Möhler, 2006a). These effects have been linked to its ability to inhibit glucocorticoid production via the HPA-axis (Herman et al., 2004), as well as influencing catecholamine secretion from the adrenal gland (Parramón et al., 1995). The presence of GABA receptors in the human adrenal (Metzeler et al., 2004) suggest that it may affect adrenal steroid production, however there is little data to confirm a direct influence of GABA on adrenal steroidogenic enzymes or hormone production in the adrenal gland. The use of herbal remedies containing GABA has been linked to potential anti-cancer properties, as well as effects on platelet aggregation and the migration and metastasis in colon carcinoma (Ortega, 2003).

The presence of GABA, L-canavanine, as well as all of the 20 naturally occurring amino acids, and D-pinitol has been confirmed in *S. frutescens* extracts by LC-MS (Mncwangi and Viljoen, 2012; Shaik et al., 2010). Collectively, the amino acids represented between 10 and 15% (w/w) of the dried plant material, of which proline, alanine and asparagine contributed approximately 60% of the total amino acid content. However, the amino acid content varies considerably between populations with not all samples containing these amino acids (Mncwangi and Viljoen, 2012). Nevertheless, GABA concentrations in the leaves of *S. frutescens* were found to be relatively high compared to other plant species as GABA is naturally low in most plants (Shelp et al., 1999). Ojewole et al. showed that *S. frutescens* extracts elicited anti-convulsant properties when seizures were induced in mice (Ojewole, 2004). Although the results were inconsistent and conclusions regarding anti-convulsant properties could not be made, the aqueous shoot extract did delay the onset of some induced seizures to a similar extent as diazepam (a well-established anti-seizure drug). The study showed that the extract acted in a GABA-like manner eliciting anti-convulsant properties (Ojewole, 2004). In addition, Tai et al. (2004) showed that the *S. frutescens* extract elicited anti-proliferative effects in several human tumour cell lines (Tai et al., 2004). The studies by Ojewole (2004) and Tai et al. (2004) attributed the effects of the extract to the presence of GABA.

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GABA typically exerts a neuro-inhibitory effect through binding to the $GABA_A$ receptor in the brain. $GABA_A$ receptors are benzodiazepine-like receptors which are functionally closely related to peripheral benzodiazepine receptors (Rudolph and Möhler, 2006b). Upon GABA or GABA agonists binding to the $GABA_A$ receptors, chloride ions are released resulting in hyperpolarization and subsequently reduced neuronal activity (Zorumski et al., 2013). The inhibitory role of $GABA_A$ receptors in the central nervous system is well documented and many compounds, including barbiturates, neurosteroids, benzodiazepines and ethanol have been shown to bind to these receptors resulting in anxiolytic effects. Adrenal steroids, specifically the neuro-active metabolites of PREG, have been shown to act as neurosteroids and influence mood, anxiety and stress responses by binding to the $GABA_A$ receptor and exerting GABA-like effects (Belelli and Lambert, 2005; Gunn et al., 2015). Although GABA generally exerts its central effects through interactions with both the $GABA_A$ (ligand-gated ion channels) and $GABA_B$ (G-protein-linked) receptors, $GABA_A$ receptors have been shown to be of greater significance with regard to the activation of the HPA-axis and stress-related effects (Brickley and Mody, 2012). The neuro-inhibitory properties of GABA allows it to influence the HPA-axis, the feedback inhibition by glucocorticoids and ultimately the stress response. These effects are mostly regulated at the PVN level enabling control of neuroendocrine activation during the stress response (Cullinan et al., 2008).

In addition to its role in neurotransmission, it has been shown that GABA exerts neuromodulatory effects in the autonomic nervous system as well as eliciting hormone-like effects in peripheral tissues. The compound has been linked to the development of endocrine disorders such as diabetes mellitus and adrenal gland diseases (Gladkevich et al., 2006). Although the expression of $GABA_A$ receptors has been observed in the bovine and rat adrenal medulla, the influence of GABA at these sites has been associated mostly with catecholamine secretion (Inoue et al., 2010; Matsuoka et al., 2008). Studies have also shown that $GABA_A$ receptors are expressed in the adrenal cortex and in H295R cells (Metzeler et al., 2004), but to our knowledge, no studies have reported the effects of GABA on steroid production in the adrenal gland (Mishunina and Kononenko, 2002). Although it is well known that GABA influences steroid output by influencing the HPA–axis (Crowley and Girdler, 2014), there is little data that suggest a local effect of GABA on steroid production.

We hypothesized that the effect of GABA on reducing anxiety and stress could be due to a localised effect on adrenal steroid production, since $GABA_A$ receptors are expressed in the adrenal gland (Metzeler et al., 2004), thus reducing elevated glucocorticoid output leading to

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reduced anxiety. We subsequently investigated the influence of GABA on steroid production by P450 enzymes in H295R cells.

Materials and methods

General

GABA, L-canavanine and DMEM was purchased from Sigma-Aldrich (RSA) while the steroids were sourced from Steraloids (Wilton, USA). Cosmic calf serum was supplied by HyClone®, Thermo Scientific (RSA). *S frutescens* subspecies were supplied by Professor Ben-Erik van Wyk, Department of Botany of Rand Afrikaans University (Voucher specimen from Mrs. Grobler: C. Albrecht s.n sub B.-E. van Wyk 4126 (JRAU)). All chemicals used were of high analytical grade and obtained from reputable suppliers.

Derivatization of GABA from *S. frutescens* extracts

The derivatization protocol was previously reported by Colling et al. and used to determine the concentration of amino acids in *S. frutescens* (Colling et al., 2010). A 50 µL aliquot of an extract (53.43 mg/mL) was transferred to a glass hydrolysis tube and dried under reduced pressure for approximately one hour. The sample was dried under reduced pressure for another hour after the addition of 20 µL methanol:water:thriethylamine (2:2:1) and the adjustment of the pH. After adding the derivatization solution, methanol:water:thriethylamine:phenylisocyanate (PITC) (7:1:1:1), the sample was incubated at room temperature for 10 minutes and then subjected to drying under reduced pressure for an hour. The sample was diluted in 200 µL 50 % methanol (v/v) solution and subjected to LC-MS/MS (Alliance HPLC coupled to a API Quatro Micro triple quadrupole mass spectrometer (Waters, Millford, MA, USA). The sample was separated using 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B) on a Waters Xbridge C18, 2.1mm×50mm, 3.5µm column. The isocratic gradient, 1 minute at 96% solvent A, was followed by a 7 minute linear gradient, 60% solvent B to 40% solvent A, whereafter the system was returned to 96% solvent A for 7 minutes. Linear standard curves, produced using 4 concentrations of GABA, asparagine, arginine and canavanine, were used to calibrate the samples.

Inhibition of steroid metabolism in H295R cells

Basal steroid metabolism in H295R cells was assessed in the presence of 10 µM GABA with and without the extract (1 mg/mL) as previously described (Schloms et al., 2012). Confluent cells were seeded into 12 well plates at $4x10^5$ cells/mL/well, and allowed to settle for 48 hours before the medium was replaced with growth medium containing 0.1 % cosmic calf serum.

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Cells were incubated for 12 hours before the addition of GABA and *S. frutescens* to the cells. Aliquots, 500 µL, were collected after 48 hours and the steroids extracted as previously described in Chapter 4. The steroid residue was resuspended in 150 µL methanol for analysis by UPLC-LCMS. Steroids were separated using a Phenomenex UPLC Kinetex PFP (2.1 mm × 100 mm, 2.6 um) column and analyzed and quantified by UPLC-APCI-MS (Schloms et al., 2012).

Cell viability assays

The viability of the H295R cells in the presence of the extract was determined using an *in vitro* cytotoxicity assay kit based on an MTT assay (Sigma-Aldrich, RSA) according to the manufacturer's instructions. Viability was assayed using $4x10^5$ cells/mL in the presence of 1 mg/mL extract or 10 µM GABA. Cell viability was not affected at these concentrations and no detrimental or stimulatory effects were observed.

Statistical analysis

Experiments were repeated in duplicate with three replicates within each experiment (unless stated otherwise). Mean values of data obtained in each experiment were analyzed using one-way ANOVA and appropriate post-tests as indicated in figure legends. The error bars represent the standard error of the mean (SEM). All data manipulations, graphical representations and statistical analysis were performed using Graph Pad Prism 5 (GraphPad Software, Inc, CA, USA).

Results

Quantification of GABA in the methanolic *S. frutescens* extract.

A sample of the methanolic extract was subjected to derivatization and separation by LC-MS/MS as described by Colling et al. (2010). The samples were analysed for amino acid content, specifically GABA, asparagine, arginine and canavanine. Although the aim of the study was to determine the concentration of GABA in the extract, the concentrations of asparagine, arginine and canavanine were also quantified (Fig 5.1) as the derivatization method has not been validated and results may vary between plant samples. The concentrations of these amino acids were subsequently used for the relative comparison to the amount of GABA present. GABA, asparagine, arginine and canavanine were quantified at ± 0.8, 1, 0.4 and 0.5 µg/µL, respectively.

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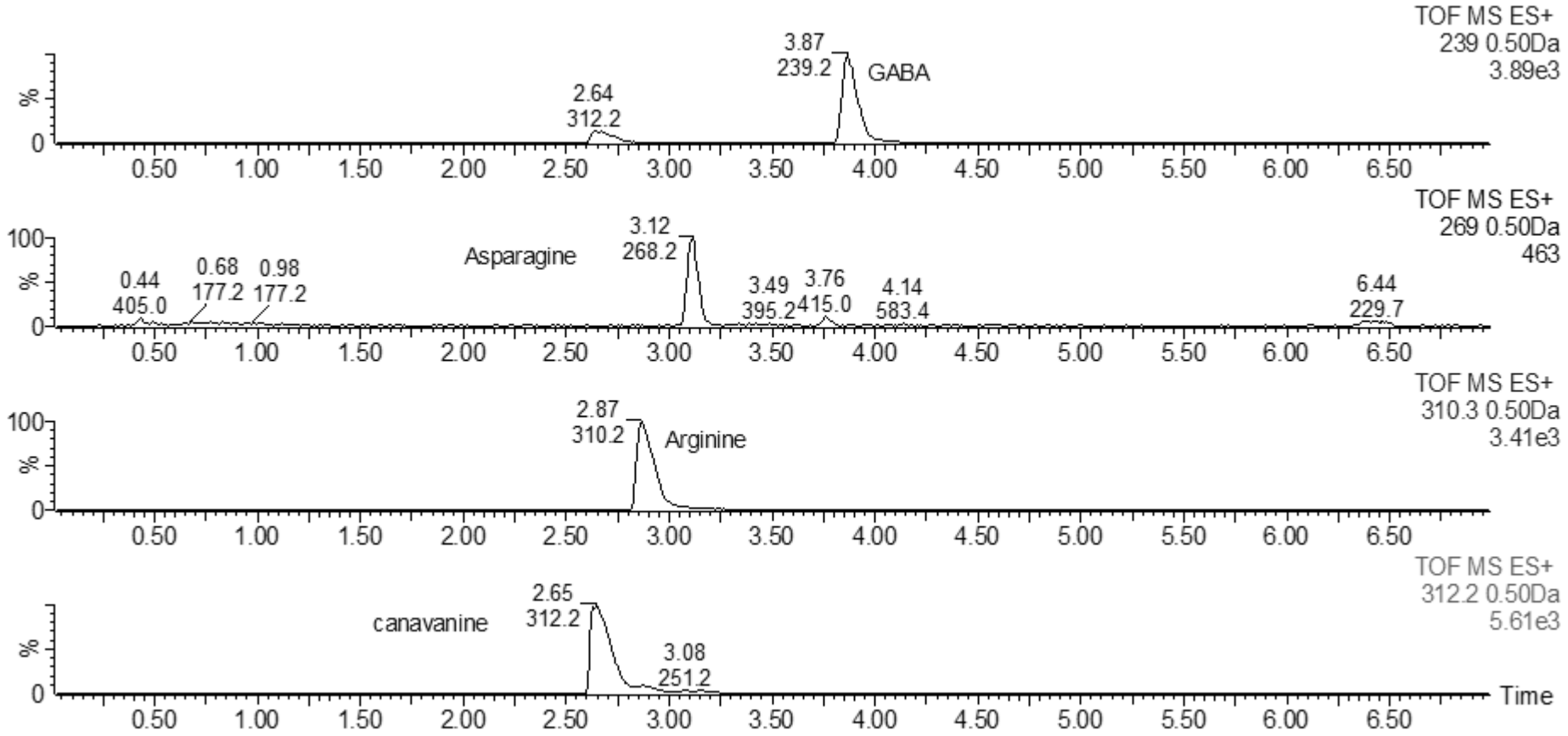

Fig. 5.1: Total ion chromatograms (ESI + mode) of the derivatised amino acids present in a methanolic *S. frutescens* extract.

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The influence of GABA on steroid production in H295R cells

The influence of GABA on adrenal steroidogenesis was investigated to determine whether the compound would ultimately affect glucocorticoid, mineralocorticoid and androgen production in H295R cells. The results in Table 5.1 show the steroid metabolites produced in H295R cells in the presence of GABA and/or a methanolic *S. frutescens* extract as compared to steroid production under basal conditions.

The presence of GABA resulted in an increase in 11-DHC (± 30 to 60 nM) levels, and decreased levels of cortisol (± 930 to 655 nM) and adrenal androgens, A4 (± 995 to 700 nM), 11OHA4 (± 195 to 125 nM), testosterone (± 50 to 30 nM), while total steroid production was reduced, ± 4040 to 3200 nM. The presence of GABA reduced total steroid production 1.3-fold, however the addition of extract together with GABA resulted in a greater reduction, 1.7-fold. In the presence of GABA, only DOC (± 7 to 13 nM) levels increased significantly, while decreased levels were detected for CORT (± 255 to 140 nM), 17OH-PROG (± 45 to 20 nM), 16OH-PROG (± 50 to 25 nM), deoxycortisol (± 1445 to 940 nM), cortisol (± 930 to 440 nM), A4 (± 995 to 575 nM), 11OHA4 (± 195 to 124 nM), and testosterone (± 50 to 20 nM).

The influence of GABA and the extract on total steroid production was similar with a decrease of 1.26- and 1.4-fold, respectively. Both conditions decreased CORT levels with the extract having a greater effect (± 236 nM vs ± 124 nM). Interestingly, while GABA did not lower DHEAS levels significantly (± 9 nM vs ± 6.5 nM), DHEAS levels were significantly increased in the presence of the extract (± 24 nM). When the effects of GABA in the absence and presence of extract were compared, the results showed that the presence of the extract further decreased total steroid production (± 3200 to 2400 nM) as well as CORT (± 235 to 143 nM) levels, while DHEAS levels decreased from 24 nM to 18 nM.

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Table 5.1: Steroid levels in H295R cells in the presence of GABA and GABA with *S. frutescens* analysed after 48 hours. The data represents the average of two experiments conducted in triplicate (n=6).

Steroid metabolite	Basal	1 mg/mL *S. frutescens*	10 µM GABA	GABA + *S. frutescens*
	% Total ± SEM	% Total ± SEM	% Total ± SEM	% Total ± SEM
PREG	ND	ND	ND	ND
PROG	0.02 ± 0	0.02 ± 0.01 ns	0.04 ± 0.01 ns	0.03 ± 0.01 ns
DOC	0.17 ± 0.02	0.47 ± 0.03 ns	0.23 ± 0.04 ns	0.55 ± 0.06 ↑ P<0.05
CORT	6.31 ± 0.6	4.3 ± 0.58 ↓ P<0.05	7.35 ± 0.95 ns	5.95 ± 1.27 ↓ P<0.01
18OH-CORT	0.33 ± 0.11	0.38 ± 0.1 ns	0.45 ± 0.1 ns	0.57 ± 0.14 ns
ALDO	0.07 ± 0.04	0.03 ± 0.02 ns	0 ± 0 ns	0 ± 0 ns
11-DHC	0.69 ± 0.03	0.9 ± 0.13 ns	1.78 ± 0.35 ↑ P<0.01	1.95 ± 0.6 ↑ P<0.01
17OH-PREG	ND	ND	ND	ND
17OH-PROG	1.13 ± 0.06	1.63 ± 0.11 ns	1.17 ± 0.05 ns	1.3 ± 0.06 ↓ P<0.05
16OH-PROG	1.19 ± 0.02	1.19 ± 0.06 ns	1.08 ± 0.09 ns	1.1 ± 0.07 ↓ P<0.01
Deoxycortisol	35.79 ± 1.76	36.89 ± 1.08 ↓ P<0.05	39.67 ± 2.13 ns	39.24 ± 0.75 ↓ P<0.01
Cortisol	23.12 ± 0.75	17.57 ± 0.58 ns	20.52 ± 0.87 ↓ P<0.001	18.45 ± 0.67 ↓ P<0.001
Cortisone	0.18 ± 0.03	0.26 ± 0.04 ns	0.16 ± 0.02 ns	0.23 ± 0.05
DHEA	ND	ND	ND	ND
DHEAS	0.23 ± 0.02	0.85 ± 0.15 ↑ P<0.001	0.17 ± 0.01 ns	0.76 ± 0.17 ns
A4	24.61 ± 0.4	28.78 ± 0.71 ns	21.94 ± 0.57 ↓ P<0.05	23.99 ± 2 ↓ P<0.01
11OHA4	4.84 ± 0.3	5.47 ± 0.3 ns	3.87 ± 0.27 ↓ P<0.01	4.95 ± 0.25 ↓ P<0.01
Testosterone	1.31 ± 0.06	1.26 ± 0.11 ns	1.02 ± 0.13 ↓ P<0.05	0.93 ± 0.15 ↓ P<0.01
Total steroid (nM)	4040 ± 78	2900 ± 135 ns	3200 ± 226 ↓ P<0.05	2400 ± 248 ↓ P<0.001

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Even though GABA in the presence of the extract decreased steroid output, the androgen production remained at 27% in the case of GABA, and 30% in the case of GABA in the presence of extract. The data suggests that GABA attenuates the effect of the extract on androgen output as 36.5% of adrenal steroids are channelled towards androgen production in the presence of the extract alone, although levels did not exceed those in basal steroid production: Basal = 30% (± 1250 nM); GABA = 27% (± 865nM); GABA + 1 mg/mL *S. frutescens* = 30% (± 732 nM) and *S. frutescens* = 37% (± 1056 nM). It is interesting to note that, as was found in the Chapter 4, the *S. frutescens* extract decreased glucocorticoid biosynthesis, while both CORT and cortisol levels were decreased by GABA which was amplified in the presence of *S. frutescens*.

Cell viability

The viability of H295R cells in the presence of GABA was assessed in a MTT cytotoxicity assay. Figure 5.2 shows that there is no significant difference in cell viability between the control and test (*S. frutescens* extract or GABA) experiments.

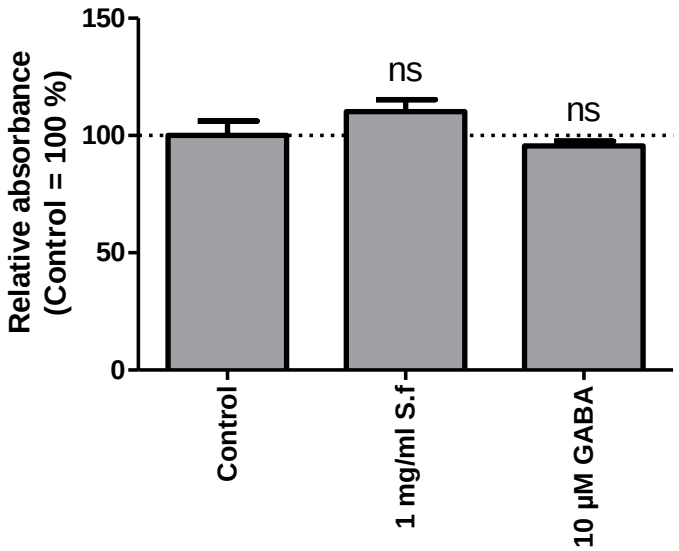

Fig. 5.2: *S. frutescens* extracts (S.f, 1 mg/mL) and GABA (10 µM) did not affect the viability of H295R cells. A one-way ANOVA, followed by a Dunnette's comparison test was used to determine significant differences (ns, non significant).

Discussion

The aim of this investigation was to determine whether GABA would influence adrenal steroidogenesis in H295R cells. The results show that the addition of GABA to H295R cells

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resulted in a significantly different steroid metabolite profile when compared to basal steroid production, with the greatest effect on adrenal androgen production.

The GABA content of the methanolic *S. frutescens* extract was determined by subjecting an aliquot of the extract to derivatization and subsequent separation by LC-MS/MS. As amino acids are polar, derivatization is employed to make the amino acids more volatile, less reactive and generally improve their chromatographic characteristics (Bidlingmeyer et al., 1984). We determined GABA levels to be 1.5 mg GABA/g dried plant material which falls between the ranges previously reported. Shaik et al. showed that leaf extracts of *S. frutescens* contained 3.48 mg GABA/g dried plant material, whereas van Wyk and Albrecht found 0.23 and 0.85 mg/g dried plant material (Shaik et al., 2010; van Wyk and Albrecht, 2008). The difference in GABA levels reported could be attributed to pH, light exposure and general growth conditions since the specimens used by Shaik et al. were grown *in vitro* and van Wyk and Albrecht utilized field specimens (Shaik et al., 2010; van Wyk and Albrecht, 2008). Shaik et al. showed that L-arginine, alanine and proline comprised approximately 60% of the total amino acid content of *S. frutescens* extracts (Shaik et al., 2010). However, previous reports indicate that the amino acid content of the extracts is highly variable — asparagine: 1.6 – 3.5 mg/g extract, arginine: 0.5 – 6.7 mg/g extract, and canavanine: 0.42 – 14.5 mg/g extract (Moshe et al., 1998; van Wyk and Albrecht, 2008). This variability can be attributed to differences in extraction methods, the geographical location of the plants and harvesting methods. Our results showing that asparagine, arginine and canavanine were present in the extract at 1.82, 0.68 and 0.90 mg/g dried plant material, respectively, are comparable to these previously reported studies. In the presence of the *S. frutescens* extract (1 mg/mL), the final concentration of GABA added to the H295R cells was approximately 140 µM. We assayed GABA at 10 µM as this concentration was used in previous studies investigating the effects of glucocorticoids on the GABAergic system in rat cortical microsacs (Strömberg et al., 2005).

Several effects elicited by *S. frutescens* extracts have been attributed to the presence of GABA, specifically anti-anxiety, anti-convulsant (Ojewole, 2004), anti-proliferative and pro-apoptotic effects (Tai et al., 2004). Recent studies have shown that disrupted endocrine systems play a major role in the development of cancers as well as cancer relapses caused by chemotherapy induced endocrine resistance (Casco and Soto-Vega, 2016). The anti-cancer effect of the extract may be linked to the extract's ability to reduce glucocorticoid production during steroidogenesis and the repression of NF-κB-driven gene transcription mediated by the GR as shown in Chapter 4. Although we have identified compounds that could contribute to these

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effects, the contribution of GABA to the reduction in steroid metabolites could not be assumed and was therefore investigated in this study. In addition, the sedative effects of the extract could be attributed to the effects of GABA on neurotransmission in the hippocampus as subjects receiving GABA orally showed the compound to have anxiolytic effects as the subjects showed decreased anxiety (Abdou et al., 2006).

The inhibitory role of $GABA_A$ receptors in the central nervous system is well documented and many compounds, including barbiturates, neurosteroids, benzodiazepines and ethanol have been shown to bind to these receptors causing anxiolytic effects. Studies have shown that PREG and DHEA metabolites exert neural excitatory effects as these neurosteroids antagonize the inhibitory effects of GABA on $GABA_A$ receptors, whereas PROG metabolites exert inhibitory effects by binding as agonists to the $GABA_A$ receptors (Falkenstein et al., 2000; Vallée et al., 2000). Although the main site of androgen production is the gonads, testosterone is also produced in the adrenal cortex (Udhane and Flück, 2016) and adrenal androgens, specifically androstanediol — a downstream metabolite of testosterone, has been shown to directly affect the $GABA_A$ receptor (Reddy and Jian, 2010). In addition, studies have shown that the phosphorylation of the GABA receptor's subunits may influence neurosteroid activity adding variability to neurosteroid responses (Comenencia-Ortiz et al., 2014).

Earlier investigations have confirmed the presence of various $GABA_A$ receptor subunits within the adrenal gland and H295R cells, however, these studies have not investigated the localized effect of GABA on steroid metabolism in H295R cells. Metzeler et al. found mRNA traces for α_2, α_3, β_2, β_3, γ_2, and ε subunits of the $GABA_A$ receptor in H295R cells, however no mRNA for α_1 and β_1 was detected (Metzeler et al., 2004). $GABA_B$ receptor subunits were also detected in these cells. Additionally, it was shown that GAD, an enzyme involved in GABA production, is active in H295R cells. The GABA receptor subunits and GAD play a significant role in the GABAergic system and the expression of these subunits and enzyme has been found to be similar in other endocrine tissues (Metzeler et al., 2004).

Our results show that GABA significantly influenced steroid production in H295R cells, decreasing adrenal androgen production to the greatest extent (1.4-fold). Although mineralocorticoid production appeared to be unchanged, the steroid shunt in the glucocorticoid pathway was decreased 1.2-fold by GABA. Glucocorticoid and adrenal androgen production were decreased by 1.7-fold and mineralocorticoid production was decreased by 1.4-fold when the extract was added to the cells incubated with GABA. The cortisol:cortisone ratio was not

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significantly affected by the presence of GABA, however the CORT:11-DHC ratio decreased. After the addition of the extract, both these ratios were decreased significantly (a trend observed in previous experiments (Chapter 4) where the extract decreased the CORT:11-DHC ratio in H295R cells). This observed trend thus suggests a further lowering effect on active glucocorticoid levels which may contribute to the anti-stress and anti-inflammatory effects of the extract. In addition, GABA decreased the testosterone:cortisol ratio, reflecting its application in mood disorders as increased testosterone:cortisol ratios have been linked to an increase in violence (Montoya et al., 2012; Romero-Martínez et al., 2013). The decrease in CORT and increase in DHEAS levels by the extract is a consistent result observed in the H295R experiments in Chapter 4. The increase in GABA concentration does not seem to alter the ability of the extract to stimulate the production of DHEAS. A similar effect was observed in Chapter 4, where SUs were unable to stimulate DHEAS production whereas the extract appeared to favour the sulfonation reaction, even under forskolin stimulated conditions. Increased levels of DHEAS may however counteract the anxiolytic effects associated with the extract as this steroid metabolite has been shown to act as an allosteric $GABA_A$ receptor antagonist (Majewska et al., 1990). The antagonistic effects of DHEAS on the $GABA_A$ receptor may however be concentration dependent as the effects elicited by this steroid have been shown to vary at high and low physiological concentrations (Reddy, 2010).

In addition to DHEAS, PREGS is also produced from PREG by SULT2A1 (Strott, 2002) and although we did not investigate the formation of PREGS in the H295R cells, we could speculate that PREGS production would also be increased in the presence of the extract as PREGS and DHEAS have both been shown to elicit excitatory effects after binding to the $GABA_A$ receptors (Paul and Purdy, 1992; Rupprecht and Holsboer, 1999) and act as $GABA_A$ receptor antagonists. Studies have shown the stress-induced downregulation of GABA1α receptors in hippocampal tissues of rats after the ingestion of *S. frutescens*, suggesting that the effect of the extract in the brain may not only be exerted through the inhibitory effect of GABA in the extract, but also the downregulation of GABA receptor expression. Previous reports confirmed decreases in GABA receptors in response to stress, however these decreases are associated with total $GABA_A$ receptor decreases and it can therefore not be assumed that the anxiolytic effect of the extract would be abolished because of the interaction with only one GABA receptor subunit (Smith and van Vuuren, 2014). As a previous report has confirmed the activity of an ethanol extract of *S. frutescens* in the GABA-benzodiazepine receptor binding assay (Stafford et al., 2005), we cannot exclude the ability of compounds within the extract to potentially bind to the

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$GABA_A$ receptor and eliciting anxiolytic effects. In addition, it has been shown that neurosteroids may bind directly to GABA receptors and elicit anxiolytic responses (Wang, 2011).

The decreased testosterone production (and perhaps the other C_{19} steroids in the androgen pathway) in H295R cells in the presence of GABA may add scientific merit to the therapeutic application of the extract as an anti-convulsant therapy as testosterone has been shown to modulate susceptibility to seizures (Reddy and Jian, 2010). Furthermore, the decrease in adrenal androgen production, specifically testosterone, in the presence of GABA may have implications in the development of prostate cancer. Traditional views that increased circulating testosterone levels are directly related to the development of prostate cancers have been questioned and there is conflicting evidence suggesting that elevated testosterone levels may not be indicative of prostate cancer. Varying results and potentially skewed study designs have all contributed to the controversy surrounding testosterone and prostate cancer (Klap et al., 2015). However, the decrease in glucocorticoids observed may suggest a potential anti-inflammatory action of GABA, when used for the treatment of inflammatory disorders, and as inflammation is associated with many cancers, this anti-inflammatory effect could add to the anti-cancer effects of GABA. However, as the present investigation has shown, it is unlikely that GABA is the major contributing compound to the anti-inflammatory and anti-cancer properties of *S. frutescens* (Gallo-Payet and Payet, 2003).

Considering the results obtained by Smith and van Vuuren showing that relatively low dosages of *S. frutescens* resulted in decreased GR expression in the hippocampus and reduced expression of the $GABA_A$ α1 receptor subunit, it is plausible that compounds within the extract facilitate the transport of GABA (and potentially other compounds) across the blood-brain barrier. The combinations of compounds present in the extract may thus impact on transport and delivery mechanisms to target sites.

In addition to GABA, the anti-anxiety effects of the extracts could also be elicited through flavonoid compounds present in the extract as studies have shown that flavonoid compounds bind the $GABA_A$ receptor with high affinity (Marder and Paladini, 2002; Medina et al., 1997). For example, flavonoid-rich orange juice extracts displayed significant agonist activity on the benzodiazepine binding site of $GABA_A$ receptors resulting in anti-epileptic and anti-convulsant effects (Citraro et al., 2016). Studies have also shown that rutin, identified within *S. frutescens* extracts, exerts anxiolytic effects in rats through interacting with $GABA_A$ receptors in the

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basolateral amygdala while these effects could not be associated with benzodiazepine receptors (Hernandez-Leon et al., 2017). Furthermore, it has been shown that flavones bind specifically to the benzodiazepine sites of the $GABA_A$ receptor with high affinity, inducing effects comparable to that of benzodiazepine (Kahnberg et al., 2002).

The altered local steroid production in the presence of GABA may be explained by the presence of $GABA_A$ receptors in the adrenal gland as GABA is known to inhibit steroid production via the HPA-axis when binding to these receptors (Crowley and Girdler, 2014; Cullinan et al., 2008). This inhibitory effect has however only been localized to the hippocampus and further investigations are required to investigate the local effect of GABA and GABA receptors on steroid production in the adrenal. The effects elicited by GABA are unlikely to be due to a direct interaction with the steroidogenic and P450 enzymes as Prevoo et al. have shown that GABA does not influence PROG and PREG binding in ovine adrenal microsomes (Prevoo et al., 2008). Furthermore, GABA requires active transport across the blood-brain barrier, which additionally suggests that a transporting mechanism could be involved in the delivery of ingested GABA at concentrations high enough to elicit the effects associated with *S. frutescens* administration (Kuriyama and Sze, 1971; Malakoutikhah et al., 2010). Numerous studies have shown that GABA is transiently absorbed in the intestine through rapid transepithelial transport which is pH-dependent. After oral administration, GABA has also been shown to induce anti-convulsant and anxiolytic effects as expected (Thwaites et al., 2000). Smith and van Vuuren have however shown that rats receiving relatively low dosages of *S. frutescens* displayed decreased GR expression in the hippocampus accompanied by a reduced expression of the $GABA_A$ α1 receptor subunit, although these effects were independent of each other (Smith and van Vuuren, 2014). The $GABA_A$ α1is generally associated with a sedative effect (Vinkers et al., 2009) and could thus account for the calming effect associated with the use of *S. frutescens*. The effect of GABA on transcriptional activities of the GR was investigated in this study, however, due to highly variable data no conclusive results were obtained (data not shown).

Taken together, the results of this study show that GABA does influence glucocorticoid and adrenal androgen production in H295R cells and in doing so may contribute to the anti-inflammatory, anti-stress and anti-anxiety properties associated with the traditional use of *S. frutescens* for the treatment of disorders associated with excess glucocorticoid production. The results suggest that *S. frutescens* could exert anxiolytic effects through rapid, non-genomic (neuro inhibition through GABA and flavonoid compounds) as well as longer term genomic effects (downregulation of GR and GABA receptors), however further investigations are

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required to confirm these mechanisms. Future investigations into the inhibitory effect of GABA on individual steroidogenic enzymes and steroid receptors may also shed light on the mechanism through which GABA influences adrenal steroid production as previous studies have shown that GABA does not bind directly to P450 enzymes.

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Chapter 6: Conclusion

S. frutescens is one of southern Africa's most popular medicinal plants and is used for a plethora of ailments and a wide range of diseases, with acquired immunodeficiency syndrome (AIDS) being the most recent addition. While its popular traditional use has been in the treatment of cancers, anecdotal evidence does suggests that *S. frutescens* extracts can be used in the treatment of depression and inflammation (van Wyk and Wink, 2004). Some scientific studies have been undertaken to investigate the molecular mechanism of action of *S. frutescens* and show that the anti-inflammatory effects of *S. frutescens,* are associated with the inhibition of the COX-2 enzyme (Na et al., 2004) and influences on cytokine activation (Gonyela, 2016; Jiang et al., 2014; Lei et al., 2014). However, studies linking the anti-inflammatory or anti-stress effects to the dysfunction of the endocrine system are limited. A study from our group has shown interactions of the extract and P450 enzymes and linked these effects to the anti-stress, anti-anxiety and anti-depression properties of the extract (Prevoo et al., 2008, 2004). The results presented in this thesis have added scientific merit to the use of *S. frutescens* extracts as anti-inflammatory and anti-stress treatments and has shown, for the first time, that the extracts may exhibit anti-hypertensive properties. Considering that MR antagonists are used in the treatment of patients with heart failure, the results suggest that *S. frutescens* may also exhibit cardioprotective properties.

The novel finding that the extracts may display anti-hypertensive and cardioprotective properties is based on the effects of the extract on mineralocorticoid production and the antagonistic effects of the extract on MR mediated transcriptional activity. The current treatment strategies for hypertension and related cardiac disorders have relied mainly on the manipulation of the production and/or activity of vaso-active hormones such as ALDO and AngII. The increase in cardiac disorders over the past decade has renewed interest in MR antagonists despite adverse side effect profiles, such as hyperkalemia, observed when steroidal MR antagonists, such as SPL and eplerenone, are used (Tamirisa et al., 2004). In contrast, clinical data suggests that the use of non-steroidal MR antagonists may reduce the risk of the development of hyperkalemia, and generally elicit less severe side effects while maintaining anti-hypertensive properties (Piotrowski, 2012).

The results presented in this thesis have shown that extracts decreased mineralocorticoid production in H295R cells, inhibited specific P450 enzymes in the mineralocorticoid pathway (most notably CYP11B2) and antagonized the effects of ALDO on MR mediated transactivation comparable to SPL. As it cannot be deduced from the results whether specific

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compounds within the extracts act as steroidal or nonsteroidal MR antagonists, it is difficult to speculate whether it would display an improved side effect profile when compared to that of SPL side effects. Although SUB elicited similar anti-hypertensive effects by antagonizing the effects of ALDO via the MR and was able to inhibit CYP11B2, SUB did not influence mineralocorticoid production in H295R cells. The structure of SUB, resembling that of adrenal steroids, may suggest that the compound may act as a steroidal MR antagonist potentially eliciting similar adverse side effects as those associated with the use of SPL. However, the fact that SUB decreases adrenal androgen production in H295R cells may suggest that the side effects may not be as severe. This hypothesis does however require further investigation into the effects of SUB on the steroidogenic enzymes involved in downstream androgen activation and metabolism. Furthermore, as some of the side effects associated with MR antagonists such as SPL have been linked to interactions with the AR (Barthelemy et al., 2014; Bonne and Raynaud, 1974; Hughes and Cunliffe, 1988), it would be interesting to investigate the effects of SUB via the AR . In terms of the cardioprotective effects, it is unlikely that the results observed in this study could be attributed, in part, to the presence of the cardenolides in the *S. frutescens* extract as the mechanism of action of these compounds involves sodium and potassium pumps (Takahashi et al., 2011). However, studies have not confirmed whether or not cardenolides exert local effects on steroid biosynthesis in the adrenal gland.

Furthermore, this study adds merit to the use of *S. frutescens* in the treatment of inflammation. Previous studies have focussed on the ability of the extract to interfere with cytokine activation, however our results show that the extract is able to influence glucocorticoid biosynthesis and GR mediated gene transcription. This study investigated the effects of the extract and SUB on adrenal glucocorticoid production and the effects of the extract and SUB on transactivation and transrepression of gene transcription mediated via the GR. Our results showed that both the extract and SUB decreased glucocorticoids produced in H295R cells, inhibit key branch point P450 enzymes (CYP17A1 and CYP21A2) and mediate SEGRA-like effects. The novel finding that the extract and SUB elicit SEGRA-like effects may provide insight into the mechanism of action of the therapeutic application of the extracts to treat inflammation without eliciting the damaging side effects associated with long term glucocorticoid use. Although the transactivation of inflammatory gene transcription mediated via the GR was first reported to be primarily associated with adverse effects of glucocorticoid therapies, while transpression mechanisms were associated with favourable ant-inflammatory effects, it has become clear that receptor interaction and downstream gene activation is far more complex and as such this

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simplistic general distinction between the two mechanisms should be interpreted with caution. Emerging research has shown that the inflammatory effects elicited via the transcriptional activities of the GR may be the result of different ratios of GR-complex conformations, i.e. monomers vs dimers, rather than merely transactivation vs transrepression (de Bosscher et al., 2016; Sundahl et al., 2015). Taking our results together with the recent report that the GR expression is downregulated in the presence of the extract (Smith and van Vuuren, 2014), it is likely that the extract and SUB could be applied successfully as an anti-inflammatory therapy as reduced GR expression together with decreased levels of glucocorticoids available for binding to the GR and the SEGRA-like effects of the extract and SUB, will decrease the likelihood of a pro-inflammatory response. However, caution should be exercised when considering the use of this medicinal plant in the treatment of inflammation as a recent study has shown that the extract may exacerbate the inflammatory response when used as a treatment in immunocompromised HIV infected patients (Africa and Smith, 2015).

The more well-known anecdotal application of *S. frutescens* relates to its ability to reduce stress, anxiety and depression. The Zulu name for the *S. frutescens*, unwele, which translates to hair, refers to the use of the plant in preventing people from pulling out their hair in distress (van Wyk, 2008). However, in traditional medicine used successfully in the treatment of mental illness, positive effects are often attributed to a placebo effect rather than active compounds producing the physiological response. It should however be noted that while *S. frutescens* contains compounds that may exhibit neurological effects, the stress response is complex, with the release of stress hormones from the adrenal gland regulated via the HPA-axis which, in turn, is regulated by many different factors (de Kloet et al., 2008). As the susceptibility of individuals to stress-related psychopathology appears to vary considerably, the development and progression of stress and depression have been linked to variations in the sensitivities and functionalities of the HPA-axis which is associated with GR sensitivity. The latter may impact on the effect of a stressor on the brain, HPA-axis feedback actions and the ultimate development of depression. The transcription of GR mediated genes are additionally regulated by factors, such as chaperone and co-chaperone proteins (Vermeer et al., 2003), which activate a short negative feedback loop after binding to the liganded GR and decreases GR signalling (Binder, 2009). The MR, however, regulates the initiation of the stress response through transcriptional activities and determines the threshold for HPA-axis activation. The functional role of MRs in the maintenance of basal steroid levels has been suggested to occur through inhibitory actions mediated through GABAergic neurons that act on the PVN of the

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hypothalamus (Berardelli et al., 2013). In addition, the MR contributes to the exertion of rapid non-genomic effects though neurotransmission and the synaptic signalling strength in the brain (de Kloet et al., 2016, 2005; Hauer et al., 2011). A recent study has also shown that GABA influenced hypertension by antagonizing MR mediated gene transcription (Downey, 2015).

GABA has been shown to modulate the immune response by affecting the $GABA_A$ receptors and have been shown to play an important role in autoimmune diseases (Bhat et al., 2010; Jin et al., 2013). Although mostly associated with neurotransmission and potential anti-cancer effects, GABA may also contribute to the anti-inflammatory effects reported for *S. frutescens* extracts. These effects are mirrored in the results of this study as it was found that GABA reduced the glucocorticoid levels in H295R cells. The reduction in glucocorticoids can also be linked to the anti-stress and anti-anxiety effects that GABA is associated with as increased glucocorticoid levels in the brain result in adverse behavioural and cognitive effects (Frodl and O'Keane, 2013). However, the effect of GABA on steroidogenesis has been mostly associated with neurotransmission affecting the central regulation of the HPA-axis.

Our study also showed that GABA significantly influenced the production of adrenal androgens. GABA decreased DHEAS production, whereas DHEAS was significantly increased in the presence of the *S. frutescens* extract. This result may be significant as it has been shown that sulfonated steroids act on GABA receptors (Paul and Purdy, 1992) and, based on our findings, it may suggest that the anxiolytic effect associated with the use of the extract may be attributed to other compounds within the extract and not GABA as previously suggested by van Wyk (van Wyk et al., 2008). As DHEA was mostly below the limit of quantification in the H295R assays, the increase in DHEAS in the presence of the extract, even under stimulated conditions, may suggest that the DHEA:DHEAS ratio is decreased by *S. frutescens*. This could have implications for fertility, potential remodelling of cardiovascular tissues and neuronal functions as a decline in DHEA has been associated with the age-related decline in DHEA:DHEAS ratios (Prough et al., 2016). The perturbation of the DHEA:DHEAS ratio has also been shown to correlate with an increased risk of cancers, especially prostate cancer (Nowell and Falany, 2006).

In addition to the observed effects of the extract and SUB on the production of specific steroid metabolites and pathways, the ratios of steroid metabolites may be indicative of other interactions and implications with the therapeutic use of the extract. The cortisol:cortisone and CORT:11-DHC ratios have been used as indicators of active circulatory glucocorticoids as well

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as potential interactions with 11βHSD2, as this enzyme inactivates cortisol and CORT to their inactive metabolites, cortisone and 11-DHC, respectively (Palermo et al., 1996; Stewart et al., 1988).

In studying the effects of *S. frutescens* on steroidogenesis in the H295R cells, the effects of the compounds did not always mimic the effects of the extract, even when considering relative concentrations of the compounds in the extracts. Although the adrenal P450 enzymes may catalyze the hydroxylation of more than one steroid, these enzymes are reported to be highly specific for their natural substrates, and thus compounds inhibiting the binding of steroid substrates to their respective enzymes will modulate subsequent hormone biosynthesis. However, studies have shown that more than one substrate may bind to the enzymes at a time (Korzekwa et al., 1998). Moreover, it has previously been shown that the triterpenoid fraction of the *S. frutescens* extracts did not induce a reverse type I spectra in the presence of PREG or PROG in ovine adrenal microsomes (Prevoo et al., 2008), suggesting that these compounds may influence the steroidogenic P450 enzymes by binding to sites other than in the active pocket. Furthermore, it has been suggested that the binding of the first substrate may increase the enzyme's affinity for the second substrate or, alternatively, faster product generation may occur when two substrates are bound to the enzyme (Hlavica and Lewis, 2001; Omura et al., 1993). Further complicating the interaction of *S. frutescens* with the P450 enzymes are reports showing that the bioavailability of certain compounds may be increased in the presence of smaller molecules (Tang et al., 2015). These findings may be applicable to *S. frutescens* where the extract could exhibit relatively high bioavailability and could thus elicit beneficial effects, as observed by Fasinu et al., in contrast to the relatively poor bioavailability shown with the SU compounds.

In some cases, compounds that have shown biological activity in simple experimental models may not exert biological effects when used in more complicated models (Williamson et al., 2005). Whether compounds elicit biological effects is highly dependent on whether these compounds can reach their intended target. As most herbal remedies are taken orally, the effects of digestion and uptake on the potential bioavailability of the compounds should be considered. Numerous studies have shown that GABA is transiently absorbed in the intestine through rapid transepithelial transport which is pH-dependent (Thwaites et al., 2000). After oral administration, GABA has also been shown to induce anti-convulsant and anxiolytic effects as expected. A recent study has shown that a single dose of 300 mg of *S. frutescens* could result in a GI concentration of extract markedly greater than the minimum concentration

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required for the inhibition of hepatic P450 enzymes, with the greatest inhibitory effect observed for CYP3A4/5 (Fasinu et al., 2013). The bioavailability, absorption and degradation of SU compounds within the extract after oral ingestion will thus affect their overall biological activities. Although the data presented suggest that SUB could have potential applications as anti-inflammatory and/or anti-hypertensive drugs, these studies were conducted in cell line models and the possible metabolism of SUB in the GI tract should thus be considered. It is plausible that SU compounds are degraded in the GI tract due to the low pH of the GI environment and the O-glycoside bond at C-25 which would be sensitive to degradation in an acidic environment. Acid hydrolysis of SUB was shown to produce an aglycone structure and D-glucose as a by-product (Fu et al., 2008; Mbamalu et al., 2017). This modification may have a significant impact on not only the bioavailability of SUB but also in terms of the effect of SUB at target sites. It is possible that cellular effects may be increased due to increased uptake into the cell and enhanced interaction at enzyme and/or receptor level. In addition, intestinal flora also exhibit glycosidic actions which would yield the aglycone of the SU compounds. The aglycones, with greater structural similarity to the steroid hormone backbone, would also impact the catalytic activity of the P450 enzymes. It has also been shown that plasma triterpenoid levels remain low after oral administration due to extensive metabolism in the GI tract, poor membrane permeability and the low solubility of the triterpenoid aglycone (Tawab et al., 2003). After ingestion, triterpenoids such as ginsenosides, have been shown to be metabolized in the gut by intestinal bacteria, often resulting in the deglycosylation of the molecule (Kim et al., 2013). Although the pre-systematic metabolism of ginsenosides may result in relatively low plasma levels, it has been shown than these deglycosilated triterpenoids are present in hepatic tissues at much higher concentrations than in plasma and other tissues (Gu et al., 2009). SU compounds may follow similar degradation and metabolic trends as ginsenosides due to the considerable structural similarity between SU compounds and ginsenosides. Considering that the bioavailability of some structurally complex compounds are increased by the co-administration of smaller molecules (Tang et al., 2015), it is plausible that the bioavailablility of the SU compounds in the *S. frutescens* extracts may likely be increased due to the presence of the simpler polyphenols also present in *S. frutescens* resulting in the complexed compounds being more stable and effective when administered in an extract than when administered as purified isolates. The smaller molecules within the extract may therefore aid the absorption of SU compounds and increase their concentrations in plasma.

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In recent years Lipinski's rule of 5 has been used as a guideline for drug development from phytochemicals as this guide can be applied to predict the bioavailability of a drug. Molecular weight, hydrophilicity and reactive chemical groups are all considered within Lipinski's theory (Lipinski, 2004). This theory would dictate that SUB would not be a good candidate for further drug development as it does not adhere to the requirements for low molecular weight, hydrophilicity and the reactive chemical groups present on this molecule. However, research has shown that many complex molecules, such as Lipitor (Pfizer), are readily available for absorption in the GI-tract in spite of scoring "low" with regard to Lipinski's theory. Adding to this, more than 40% of oral drugs currently commercially available are poorly soluble and several measures can be implemented to increase bioavailability, including crystal engineering, micronization, nanosizing and the addition of cyclodextrins (Krishnaiah, 2010). Triterpenoids have previously been subjected to chemical modification to increase bioavailability and to increase favourable effects associated with these compounds, specifically the anti-inflammatory and anti-tumour effects (Parra et al., 2014). These modifications appear to be mostly associated with the acylation of the hydroxyl groups at C3 and C28. The biological activity of steroid hormones is also very susceptible to modifications at C3 and although the manipulation of SUB at C3 may have implications for improved bioavailability as well as associated therapeutic effects, it may alter steroidogenic enzyme as well as receptor interaction. Since the keto or hydroxyl group of the steroid hormones are of critical importance in the orientation and binding in the active pocket of p450 enzymes and thus to enzymatic activity, changes at C3 of the triterpenoid may result in loss of inhibitory effects and perhaps receptor interaction, particularly if the aglycone SUs bind in the active pocket.

The commonality between herbal remedies is that these remedies are anecdotally used to treat several different disorders with anti-inflammatory, anti-oxidant and anti-cancer properties being described most frequently (Na et al., 2004; Skerman et al., 2011; van Wyk and Wink, 2004; Venkatesha et al., 2016). These disorders are often inter-linked and the complexity of the pathogenesis often overlaps due to several factors influencing the disorder. Although studies investigating the effects of botanicals on endocrine disruption as a hypothesis for their application in the anecdotal treatments have been limited, the studies in this thesis was focussed on determining direct effects on steroid production or site-specific actions via steroid receptors. As many unbound circulatory steroids, considered to be free and therefore active steroids due to their interaction at the target site, are still used as disease indicators, the effects of herbal remedies on the binding of active steroids to steroid transporters within the bloodstream could

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be significant as the compounds may be competing with endogenous active steroids for binding sites on the steroid transporters. Studies investigating disease alleviating effects of medicinal plants have focussed on either steroid production, steroid receptor binding and downstream transcriptional activities, although the latter two have been the more popular topics. To our knowledge, no studies have investigated the potential effects of botanicals on steroid transporters within the circulatory system. In plasma, active steroids are bound to albumin, CBG and SHBG (Siiteri et al., 1982). Albumin is present in greater concentrations than CBG or SHBG, however the affinity of albumin for steroids is relatively low. In addition, circulating ALDO is mainly bound to albumin, whereas glucocorticoids and PROG are bound primarily to CBG and the androgens and estrogens are bound to SHBG (Strel 'chyonok and Avvakumov, 1990). The effect of herbal remedies, be it whole extracts or specific compounds, on steroids binding to their transporters is thus of great importance. In this context, the anti-glucocorticoid effect elicited by SUB and *S. frutescens*, as confirmed in this study, might be abolished if compounds displace glucocorticoids from CBG resulting in elevated free glucocorticoid levels in circulation. The latter will however be dependent on factors such as the serum levels of the glucocorticoids, SUB and *S. frutescens*, as well as the affinity of the transporters for the ligands. However, due to the non-specific nature of albumin binding which allows it to bind to a host of phytochemicals thus reducing potential endocrine disruptive effects (Baker, 2002), it is more likely for hydrophobic compounds in the extract, such as SUB, to be transported by albumin. In the study of therapeutic applications of herbal remedies this aspect has been severely neglected and remains to be investigated.

Although the *in vivo* biological activity of the extract and SUB was not addressed in this study, the *in vitro* results obtained indicate that *S. frutescens* extracts display anti-stress, anti-inflammatory and anti-hypertensive properties and therefore adds merit to the anecdotal use of this medicinal plant in the treatment of stress, anxiety and inflammation.

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Chapter 7: References

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Addendum 1: *Sutherlandia frutescens* modulates adrenal hormone biosynthesis, acts as a selective glucocorticoid receptor agonist (SEGRA) and displays anti-mineralocorticoid properties

The contributions to the paper were as follows:

- CA Sergeant conducted the experiments in the study.
- D Africander is the co-supervisor of PhD candidate.
- P Swart is the holder of the student bursary.
- AC Swart is the supervisor of the PhD candidate.

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Sutherlandia frutescens modulates adrenal hormone biosynthesis, acts as a selective glucocorticoid receptor agonist (SEGRA) and displays anti-mineralocorticoid properties

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ABSTRACT

Ethnopharmacological relevance: *Sutherlandia frutescens* is a traditional African medicinal plant used in the treatment of stress and anxiety, while also exhibiting anti-inflammatory properties.

Aim of study: The study aimed at linking anti-stress and anti-inflammatory properties of *S. frutescens* to its influence on glucocorticoid biosynthesis and the inflammatory response via steroid receptor interaction.

Materials and methods: The influence of *S. frutescens* extracts and sutherlandioside B (SUB),10 and 30 μM, on key steroidogenic enzymes was assayed in COS-1 cells. Effects were also assayed on basal and stimulated hormone levels in the adrenal H295R cell model. Agonist activity for transactivation and transrepression of the extract and SUB with the glucocorticoid- (GR) and mineralocorticoid receptor (MR) was subsequently investigated.

Results: Inhibitory effects of the extract towards progesterone conversion by CYP17A1 and CYP21A2 were significant. SUB inhibited CYP17A1 and 3β-HSD2, while not affecting CYP21A2. In H295R cells, SUB decreased cortisol and androgen precursors significantly. The extract decreased total steroid production (basal and stimulated) with cortisol and its precursor, deoxycortisol, together with mineralocorticoid metabolites significantly decreased under forskolin stimulated conditions. *S. frutescens* extracts and SUB repressed NF-κB-driven gene expression without activating GRE-driven gene expression and while neither activated MR mediated gene transcription, both antagonized the effects of aldosterone via the MR.

Conclusion: Data provide evidence linking anti-stress, anti-inflammatory and anti-hypertensive properties of *S. frutescens* to inhibition of steroidogenic enzymes and modulation of adrenal hormone biosynthesis. Findings suggesting *S. frutescens* and SUB exhibit dissociated glucocorticoid characteristics underline potential therapeutic applications in the treatment of inflammation and hypertension.

1. Introduction

Sutherlandia frutescens (S. frutescens) is a medicinal plant indigenous to South Africa and has customarily been used to treat a broad spectrum of ailments such as cancers, hence the common name Cancer bush, symptoms of anxiety and stress, inflammation as well as diabetes (van Wyk, 2008; Van Wyk, 2015). A number of secondary metabolites, including the sutherlandin flavonoids and triterpenoid compounds, have been isolated from *S. frutescens* extracts prepared from leaves (Fu et al., 2008), seed pods (Albrecht et al., 2012) and commercially available capsules (Avula et al., 2010). The triterpenoids identified in *S. frutescens* extracts include four complex cycloartane glycosides – sutherlandiosides (SU) A, B, C and D with SUB being the major triterpenoid present in plant material (Fu et al., 2008). The presence of the SU compounds were later also confirmed by liquid chromatography-mass spectrometry (LC-MS) (Albrecht et al., 2012; Avula et al., 2010). In the isoprenoid biosynthesis pathway of the triterpenoids, these molecules are oxidized by various cytochrome P450-dependent (P450) monooxygenases, after which glycosylation yields compounds containing a β-D-glucopyranose moiety at C24 (Lambert et al., 2011). Studies have shown that triterpenoids exhibit anti-cancer and anti-inflammatory properties with data indicating that many of these compounds target and downregulate nuclear factor-kappa B (NF-κB) (Bai et al., 2016; Chen et al., 2015; Yadav et al., 2010). Triterpenoids are furthermore widely used in Asian medicine in the treatment of chronic diseases which include amongst others, diabetes, obesity, cardiovascular atherosclerosis, arthritis and depression (Sanna et al., 2015; Venkatesha et al., 2016; Yadav et al., 2010).

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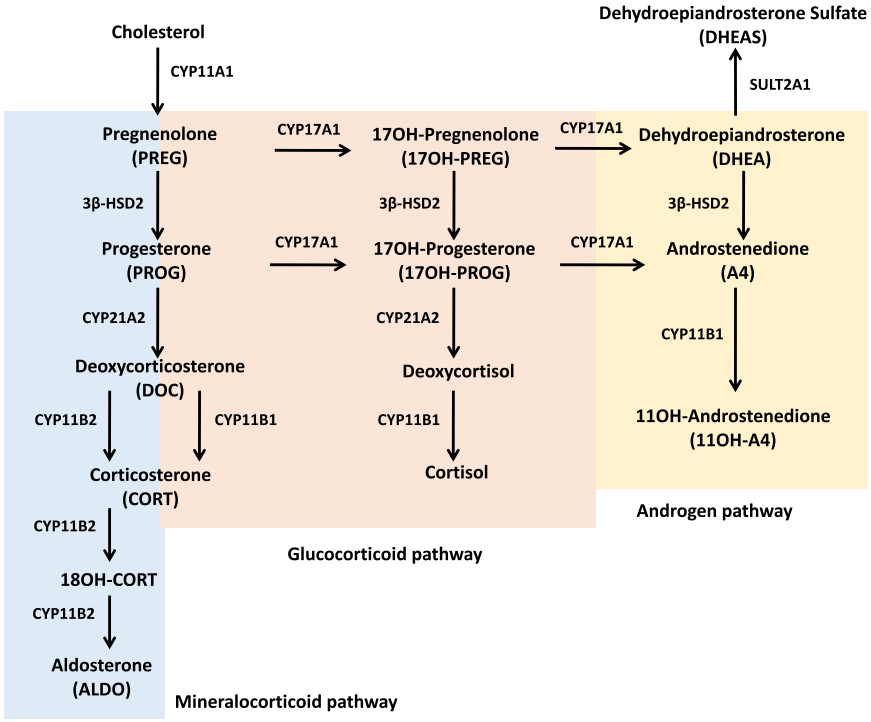

Fig. 1. Human adrenal steroid biosynthesis. The metabolism of cholesterol by the major steroidogenic enzymes yielding ALDO, cortisol and 11OHA4 in the mineralocorticoid-, glucocorticoid-, and androgen pathways, respectively.

The aforementioned conditions are closely associated with an impaired endocrine system and as such, the triterpenoids which have molecular structures closely related to the cyclopentanoperhydrophenanthrene structure of steroid hormones, may also interact with the steroidogenic P450 enzymes which catalyze steroid biosynthesis. In addition, the triterpenoids may mimic or antagonize the downstream actions of endogenous steroid hormones. While we have shown that *S. frutescens* extracts inhibit the catalytic activity of the adrenal steroidogenic P450 enzymes, P450 17α-hydroxylase/17,20-lyase (CYP17A1) and P450 21-hydroxylase (CYP21A2) (Prevoo et al., 2004, 2008), a more recent *in vivo* study by Smith and van Vuuren, 2014, showed that *S. frutescens* extracts affected steroidogenesis increasing plasma corticosterone levels in male Wistar rats (Smith and van Vuuren, 2014). Although the inhibition of several hepatic P450 enzymes by *S. frutescens* extracts has also been shown (Fasinu et al., 2013), reports on the interaction of the SU compounds with adrenal steroidogenic P450 enzymes are limited.

Adrenal P450 enzymes catalyze the biosynthesis of the steroid hormones in the mineralocorticoid, glucocorticoid and androgen pathways (Fig. 1). These steroids are all derived from the precursor steroid, cholesterol, with P450 side-chain cleavage (CYP11A1) catalysing its conversion to pregnenolone (PREG). In the mineralocorticoid pathway, limited to the zona glomerulosa of the adrenal cortex, PREG is converted by 3β-hydroxsteroid dehydrogenase type 2 (3β-HSD2) to progesterone (PROG) and subsequently to deoxycorticosterone (DOC) by CYP21A2. DOC is converted to aldosterone (ALDO) via corticosterone (CORT) and 18-hydroxycorticosterone (18OH-CORT) by aldosterone synthase (CYP11B2). In the glucocorticoid pathway, 17-hydroxypregnenolone (17OH-PREG), a product of the CYP17A1 conversion of PREG, is converted to cortisol via 17-hydroxyprogesterone (17OH-PROG) and deoxycortisol, catalyzed by 3β-HSD2, CYP21A2 and P450 11β-hydroxylase (CYP11B1) respectively. In the androgen pathway, dehydroepiandrosterone (DHEA), the product of 17OH-PREG, is converted to androstenedione (A4) by 3β-HSD2. We recently showed that A4 is also a substrate for CYP11B1 which catalyzes the formation of 11-hydroxyandrostenedione (11OHA4) (Swart et al., 2013). Although the adrenal P450 enzymes may catalyze the hydroxylation of more than one steroid, these enzymes are reported to be highly specific for their natural substrates, and compounds inhibiting the binding of steroid substrates to their respective enzymes will modulate subsequent hormone biosynthesis.

In adrenal steroidogenesis CYP17A1, together with 3β-HSD2 and CYP21A2, play a key role in channeling PREG into the three respective pathways (Goosen et al., 2013). We have previously reported that an aqueous extract of *S. frutescens* inhibited both PREG and PROG conversion by CYP17A1 significantly with the inhibition of PROG conversion being greater. The extract was also able to bind to microsomal P450 enzymes eliciting a reverse type I spectrum, suggesting that compounds bind to sites other than the active pocket. In addition, we showed that a methanolic extract and a mixture of the triterpenoid compounds inhibited the binding of PREG and PROG to adrenal microsomal P450 enzymes (Prevoo et al., 2008). Interference of the binding of PROG and PREG and the modulation of their metabolism by CYP21A2 and CYP17A1 may thus impact on the downstream production of glucocorticoids and mineralocorticoids, and in so doing also impact on the expression of various genes influenced by adrenal hormone levels via the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in target tissue. The GR and MR are generally associated with the regulation of inflammatory (Nixon et al., 2013) and hypertensive processes (Messaoudi et al., 2012), involving gene transcription which is influenced by many other factors such as co-regulators and genetic modifiers (Bosscher et al., 2016; Griekspoor et al., 2007; Harris et al., 2013).

Conventional drugs used in the treatment of inflammation and hypertension target the GR and MR, respectively. Considering that *S. frutescens* is used as a traditional medicine to treat an array of conditions and has been shown to elicit immunomodulating effects (Zhang et al., 2014), the question arises whether *S. frutescens* and the triterpenoids can act via these receptors. Although a previous report has shown that *S. frutescens* extracts down-regulate lipopolysaccharide-induced NF-κB activity (Ajit et al., 2016), to our knowledge, no previous studies have investigated the effects of *S. frutescens* or the SUs on any members of the steroid receptor family. Compounds such as ginsenosides, which are classed as triterpene saponins with an

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aglycone skeleton structure similar to the SUs, have however been shown to be functional ligands of the GR (Du et al., 2011; Lee et al., 1997). Similarly, the pentacyclic triterpene, ursolic acid, enables nuclear translocation of the GR without affecting glucocorticoid response element (GRE)-driven gene transcription (Kassi et al., 2009). While the binding of triterpenes to the GR suggests that these compounds, and possibly other structurally similar triterpenes, may modulate GR-dependent signalling pathways, studies reporting interaction of triterpenoids with the MR are limited. Saireito, a Japanese herbal medicine currently used clinically in the treatment of water retention and inflammatory edemas, comprises of a range of ginsenosides as well as the more complex saikosaponin compounds. These inhibit ALDO binding to the MR with saikosaponin H being the most potent inhibitor. The data suggests that the diuretic action of Saireito may possibly be attributed to the antagonistic effects of saikosaponin H as well as the effects of other triterpenoid saponin compounds towards the MR (Hattori et al., 2006).

The aim of this study was to investigate whether the anti-stress and anti-inflammatory properties of *S. frutescens* could be attributed to the modulation of adrenal hormone biosynthesis and interaction with the GR and the MR. We therefore determined the influence of *S. frutescens* on adrenal steroidogenesis by assessing the interaction of a methanolic extract of *S. frutescens* and SUB, one of the major SU compounds in *S. frutescens* extracts, with the enzymes in the glucocorticoid and mineralocorticoid pathways, and to investigate the influence on the steroid flux in the H295R adrenal cell model. The transcriptional activation and repression of the extract and SUB via the GR, as well as its relative agonist and antagonist properties for transactivation via the MR were also investigated to determine possible downstream effects of *S. frutescens* at the cellular level.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA), 9-α-fluoro-16-α-methyl-prednisilone (dexamethasone (Dex)), spironolactone and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Steroids were purchased from Steraloids (Wilton, USA). COS-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's phosphate buffered saline, along with penicillin-streptomycin and trypsin-EDTA were sourced from Gibco-BRL (Gaithersburg, MD, USA). *Mirus TransIT®*- LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Fetal calf serum was supplied by Highveld Biological (Lyndhurst, RSA), while cosmic calf serum was purchased from HyClone®,Thermo Scientific (South Logan, Utah, USA). *S. frutescens* subspecies (L.) R. Br. were supplied by Professor Ben-Erik van Wyk, Department of Botany of Rand Afrikaans University (Voucher specimen from Mrs. Grobler: C. Albrecht s.n sub B.-E. van Wyk 4126 (JRAU)). Triterpenoids were supplied by Prof J. Syce (University of Western Cape, Cape Town, South Africa) and Prof WR Folk (University of Missouri, Missouri, USA). All chemicals used were of high analytical grade and obtained from reputable suppliers.

2.2. Preparation of the methanolic extract of S. frutescens and SUB

S. frutescens was subjected to organic extraction using a glass soxhlet extractor coupled to a double wall condenser. Dried plant material, 18.5g, was extracted with chloroform, 250 mL, for 8 h, after which the solvent was replaced with methanol, 250 mL, and extracted for a further 8 h (Swart et al., 1993). The methanol extract was dried at room temperature at reduced pressure on a rotary evaporator. The residue was resuspended in 35 mL de-ionized water and centrifuged at 6000*g* for 5 min. The supernatant, at a final concentration of 53.43 mg/mL, was stored at −18 °C until use. LC–MS/MS analysis of the extract was conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Milford, MA, USA) using a Waters BEH C18 column (2.1×50 mm, 1.7 μm). Solvent A consisted of 0.1% formic acid in water and solvent B consisted of acetonitrile. A linear gradient from 100% A to 78% A in 3 min, followed by linear gradient from 78% A to 55% A in 7 min and a linear gradient from 55% A to 0% A in 2 min was applied. The column was returned to 100% A in 1 min, after an isocratic elution at 100% B for 2 min. The samples, 5 μl, were analysed at a flow rate of 0.35 mL/min with a total run time of 15 min for each sample. Ionization was achieved with an electrospray source with the cone voltage set at 15 V and capillary voltage at 3 kV. Analysis was carried out utilizing positive and negative modes.

S. frutescens triterpenoids were fractionated on a semi-preparative Novapak HR C18 HPLC column (6 μm spherical particles, 300 mm×7.8 mm; Millipore-Waters, La Jolla, USA) utilizing an elution gradient from 80% solvent A (0.1% trifluoroacetic acid in analytical quality water, *v*/*v*) and 20% solvent B to 70% solvent B (90% acetonitrile and 10% A, *v*/*v*) and eluted over 23 min. SUB was collected, lyophilized and dissolved in ethanol, 2 mg/mL, prior to storage at −18 °C.

2.3. Steroid conversion in transiently transfected COS-1 cells

The influence of *S. frutescens* extract and SUB on recombinant enzymes, 3β-HSD2, CYP17A1 and CYP21A2 was determined in non-steroidogenic COS-1 cells as described previously (Schloms et al., 2012). Briefly, confluent cells were plated into 12 well plates, 1×10^5 cells/mL/well, 24 h prior to transfection. Cells were transiently transfected with 0.5 μg DNA and 1.5 μl *Mirus TransIT®*- LT1 transfection reagent, used as instructed by the manufacturer. Cells were incubated for 72 h after which 1 μM of the appropriate steroid substrate was added to the medium. PREG and PROG were added to cells expressing the baboon CYP17A1 cDNA construct; PREG was added to cells expressing the baboon 3β-HSD2 construct; PROG was added to cells expressing the bovine CYP21A2 construct. The effect of the methanolic extract of *S. frutescens* on steroid conversion by these enzymes was determined by adding 2.6 mg/mL extract to the medium, and the effect of SUB by adding the compound to a final concentration of either 10 or 30 μM. Control transfection reactions were performed using the pCIneo vector, without insert DNA. Aliquots, 500 μl, were removed after 4 h and the steroids extracted with dichloromethane (10:1), vortexed for 30 s and centrifuged at 500*g* for 5 min. The organic phase was evaporated using N_2, resuspended in 120 μl methanol and stored at 4 °C prior to steroid analysis. Metabolites were separated by UPLC (ACQUITY UPLC, Waters Corporation, Milford, USA) using a Waters UPLC BEH C18 column as previously described and a Xevo triple quadrupole mass spectrometer (Waters Corporation, Milford, USA) was used for quantitative mass spectrometric detection. A linear least squares regression (weighted (1/x2)) was utilized to construct curves for calibration and the MassLynx 4.0 software program was used for data collection (Schloms et al., 2012) and steroid analyses presented as the mean, n=3.

2.4. Steroid metabolism in H295R cells

The influence of *S. frutescens* extract and SUB on steroid metabolism was assayed in H295R cells under basal and forskolin stimulated conditions as previously described (Schloms et al., 2012). Briefly, confluent cells were seeded into 12 well plates, 4×10^5 cells/mL/well, and allowed to attach for 48 h after which the medium was replaced with growth medium containing 0.1% cosmic calf serum. Cells were incubated for 12 h after which extract (1 mg/mL) or SUB (10 or 30 μM) was added, with and without forskolin, to determine the effects on basal and stimulated steroid production. Aliquots, 500 μl, were collected after 48 h and the steroids extracted as previously described. The steroid residue was resuspended in 150 μl methanol for analysis by

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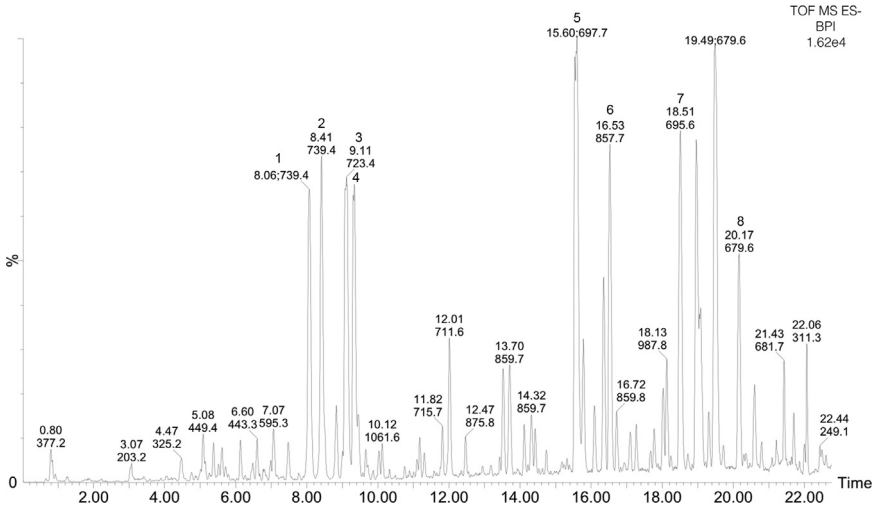

Fig. 2. Total ion chromatogram of the LC-MS analysis (ESI-) of a methanolic extract of *S. frutescens*. Retention times and masses are indicated at peaks 1–4 representing sutherlandins A-D and peaks 5–8 representing SU B, C, A and D, respectively.

UPLC-LCMS. Steroids were separated using a Phenomenex UPLC Kinetex PFP (2.1 mm×100 mm, 2.6 μm) column, analysed and quantified by UPLC-APCI-MS (Schloms et al., 2012) as described above.

2.5. Transactivation and transrepression assays

The effects of the extract and SUB on the GR were assayed in COS-1 cells which were plated in complete medium (DMEM containing 10% fetal calf serum and 1% penicillin- streptomycin) at a density of 2×10^6 cells/mL in 10 cm culture dishes. After 24 h the cells were transiently transfected with the appropriate expression vectors, using a *Trans*IT®-LT1 transfection reagent (Mirus, USA) according to the manufacturer's instructions. For transactivation assays, 900 ng pRS-hGR and 9 μg pTAT-2xGRE-Elb-luciferase, driven by the E1b promoter containing two copies of the rat TAT-GRE (Sui, 1999) were used, while for transrepression assays 1.5 μg pRS-hGR and 3 μg of the IL6-luc-promoter reporter construct (p(IL6κB)350hu. IL6Pluc+) were used. After 24 h, the transfected cells were replated into 24 well plates at a density of 5×10^4 cells/well and incubated for 24 h. The cells were washed with phosphate buffered saline (PBS) and incubated for 24 h with serum-free medium containing dexamethasone (Dex) (10 μM), *S. frutescens* (0.5 or 0.75 mg/mL) and SUB (10 or 30 μM) (for transactivation) or serum-free medium containing PMA in the absence and presence of Dex (10 μM), extract (0.5 or 0.75 mg/mL) and SUB (10 or 30 μM) (for transrepression).

The effects of the extract and SUB on the MR were assayed in CHO cells stably transfected with the rat MR, 11β-HSD2 and a pTAT3-gLuc reporter gene (Morita et al., 1996). Cells were seeded at a density of 2.5×10^4 cells/mL/well in 12 well plates in complete growth media (DMEM/F-12 containing 10% cosmic calf serum, 1% penicillin-streptomycin and 0.1% gentamicin) and incubated for 48 h. The cells were incubated for 24 h in experimental medium (DMEM/F-12 containing 0.1% charcoal stripped cosmic calf serum, 1% penicillin-streptomycin and 0.1% gentamicin) containing ALDO (10 nM), *S. frutescens* (0.5 or 0.75 mg/mL) and SUB (10 or 30 μM) to investigate agonist activity, and containing 10 nM ALDO in the absence and presence of *S. frutescens* (0.5 or 0.75 mg/mL), SUB (10 or 30 μM) or spironolactone (1 μM) to investigate antagonist activity.

Lysed cells were analysed as previously described (Africander et al., 2011) for all the reporter assays. The luciferase activity, measured in relative light units (RLUs), was normalized to protein concentration as determined using a Pierce® BCA Protein Assay Kit (Rockford, IL, USA) to correct for plating efficiency.

2.6. Cell viability

The viability of the COS-1, H295R and CHO cells in the presence of *S. frutescens* extract and SUB was determined using an *in vitro* cytotoxicity assay kit based on an MTT assay (Sigma-Aldrich) according to the manufacturer's instructions. The concentrations of cells used for these assays were 1×10^5 cells/mL for COS-1 cells, 4×10^5 cells/mL for H295R cells and 2.5×10^4 cells/mL for CHO cells. SUB was assayed at 10 and 30 μM and the extract was assayed at 0.5, 0.75, 1 and 2.6 mg/mL. Cell viability was not affected at these concentrations and no detrimental or stimulatory effects were observed. In addition, the 17-dehydrogenase activity endogenous to COS1 cells was not affected by *S. frutescens* with the conversion of testosterone to A4 being ± 90% in the presence of the extract (2.6 mg/mL).

2.7. Statistical analysis

Experiments were repeated in duplicate with three replicates within each experiment (unless stated otherwise). Mean values of data obtained in each experiment were analysed using one-way ANOVA and appropriate posttests (as indicated in figure legends). The error bars represent the standard error of the mean (SEM). All data manipulations, graphical representations and statistical analysis were performed using Graph Pad Prism 5 (GraphPad Software, Inc, CA, USA).

3. Results

3.1. Analysis of the methanolic extract of S. frutescens and SU compounds

The *S. frutescens* extract was analysed in both electronspray ionization (ESI) negative and positive mode, with a clearer visualization of the sutherlandins (1–4) and SU compounds (5–8) being obtained in ESI negative mode (Fig. 2), depicted in the total ion chromatogram (TIC). The sutherlandin and SU compounds exhibited a similar profile as previously reported with the former compounds eluting first followed by SUB, SUC, SUA and SUD. SUA and B were collected upon fractionation of a mixture of SU compounds and analysed by LC-MS in ESI positive negative (Fig. 3A) and negative (Fig. 3B) modes with the resulting ions correlating to that previously described (Albrecht et al., 2012). While both compounds have the same exact mass, 652.41, the fragmentation patterns of SUA (not shown) and SUB are also identical with the following extracted ions being

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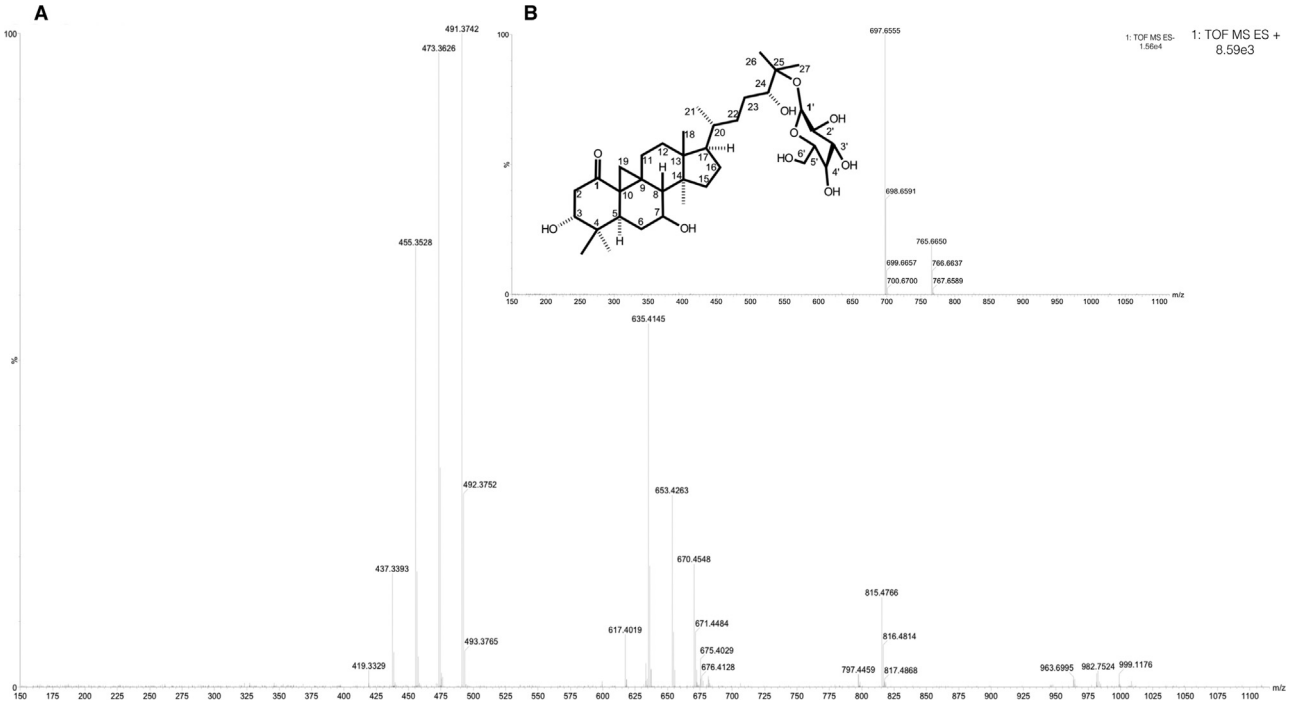

Fig. 3. LC-MS chromatogram of SUB in (A) ESI$^+$ mode and (B) ESI$^-$ mode and SUB structure (insert). In ESI$^+$ mode m/z 635.4281 $[M+H-H_2O]^+$; m/z 491.3741 $[M+H-glu]^+$; m/z 473.3635 $[M+H-glu-H_2O]^+$; m/z 455.3532 $[M+H-glu-2H_2O]^+$; m/z 437.3417 $[M+H-glu-3H_2O]^+$ and m/z 419.3275 $[M+H-glu-4H_2O]^+$ were detected. In ESI$^-$ mode the major ions detected was m/z 697.4161 $[M+formate]^-$ and 653.4281 $[M+H]^+$.

identified in ESI positive mode m/z 653.4281 $[M+H]^+$; m/z 635.4281 $[M+H-H_2O]^+$; m/z 491.3741 $[M+H-glu]^+$; m/z 473.3635 $[M+H-glu-H_2O]^+$; m/z 455.3532 $[M+H-glu-2H_2O]^+$; m/z 437.3417 $[M+H-glu-3H_2O]^+$ and m/z 419.3275 $[M+H-glu-4H_2O]^+$. In ESI negative mode the major ions previously reported for SUA and B were m/z 651.4080 $[M-H]^-$ and m/z 697.4161 $[M+formate]^-$, respectively (Albrecht et al., 2012). However, in our study m/z 697.4161 $[M+formate]^-$ was detected, but not m/z 651.4080 $[M-H]^-$.

Quantification of the major SU compounds in the methanol extract showed SUB was present at 3 mg/g *S. frutescens* dried plant material and SUA, SUC and SUD at 2.3, 1.8 and 2.6 mg/g, respectively. We subsequently focused our bioactivity studies on SUB and determined the effects of SUB on the catalytic activity of the steroidogenic enzymes at the branch point of adrenal hormone biosynthesis.

3.2. Substrate conversion by CYP17A1, CYP21A2 and 3β-HSD2 in COS-1 cells

The influence of the *S. frutescens* extract and SUB on the catalytic activity of CYP17A1 and 3β-HSD2 towards PREG, and on that of CYP17A1 and CYP21A2 towards PROG was investigated. The extract had no significant inhibitory effect on PREG conversion (Fig. 4A) by CYP17A1 however, PROG conversion (Fig. 4B) was inhibited significantly ($P < 0.001$). While the conversion of both PREG and PROG by CYP17A1 was inhibited significantly ($P < 0.01$) in the presence of SUB (Fig. 4A and B), it was only the catalytic activity towards PROG that was also significantly inhibited at the lower concentration ($P < 0.05$). The catalytic activity of CYP21A2 towards PROG (Fig. 4C) was inhibited by the extract ($P < 0.001$), however, the enzyme was not influenced by SUB. In contrast, 3β-HSD2 was inhibited significantly ($P < 0.01$) at both SUB concentrations of SUB while the extract had no inhibitory effect on the catalytic activity of 3β-HSD2 towards PREG (Fig. 4D).

3.3. Steroid metabolite production in H295R cells

Having assessed the inhibitory effects of the extracts and SUB on the branch point steroidogenic enzymes, the influence of *S. frutescens* on steroidogenesis in the H295R cell line was subsequently determined. This model system lends itself to investigations into the effects of compounds, natural products and endocrine disruptors on steroidogenesis as H295R cells express all the steroidogenic enzymes catalysing the biosynthesis of the mineralocorticoids, glucocorticoids and adrenal androgens. In addition, a stress response can be induced by exposure to forskolin which mimics the effects of ACTH via the activation of adenyl cyclase (cAMP) pathways in adrenal cells (Rainey et al., 1994).

The effects of SUB on hormone levels was firstly assayed under basal conditions to determine whether the compounds would modulate the steroid flux due to their inhibition of CYP17A1 and 3β-HSD2. Although not significant, total steroid production decreased ± 1.25-fold in the presence of both concentrations (Table 1). A4, 11OH-A4, 16OH-PROG and cortisol were significantly decreased ($P < 0.05$) and while 11-dehydrocorticosterone (11-DHC) was significantly increased ($P < 0.05$) in the presence of 30 μM SUB only the former two steroid metabolites were increased at the lower concentration.

The influence of the extract (1 mg/mL) was subsequently assessed on basal and forskolin-stimulated steroid production. Effects on basal hormone levels (Table 2) were similar to that of the compounds, with a ± 1.4-fold reduction in total steroid output, with most of the steroid levels being affected and the Δ5-steroid levels remaining below the limit of detection. DHEAS levels however, increased significantly ($P < 0.01$). 16OH-PROG levels were decreased significantly ($P < 0.01$) as in the case of SUB, indicating inhibition of CYP17A1 as this hormone is not metabolized further in the adrenal. Steroids also decreasing significantly were deoxycortisol ($P < 0.01$) and cortisol ($P < 0.001$) in the glucocorticoid pathway, as well as CORT ($P < 0.01$), while its precursor DOC, increased significantly ($P < 0.001$).

The effects of *S. frutescens* on forskolin-stimulated steroid production (Table 3) was far more pronounced. Steroid production increased

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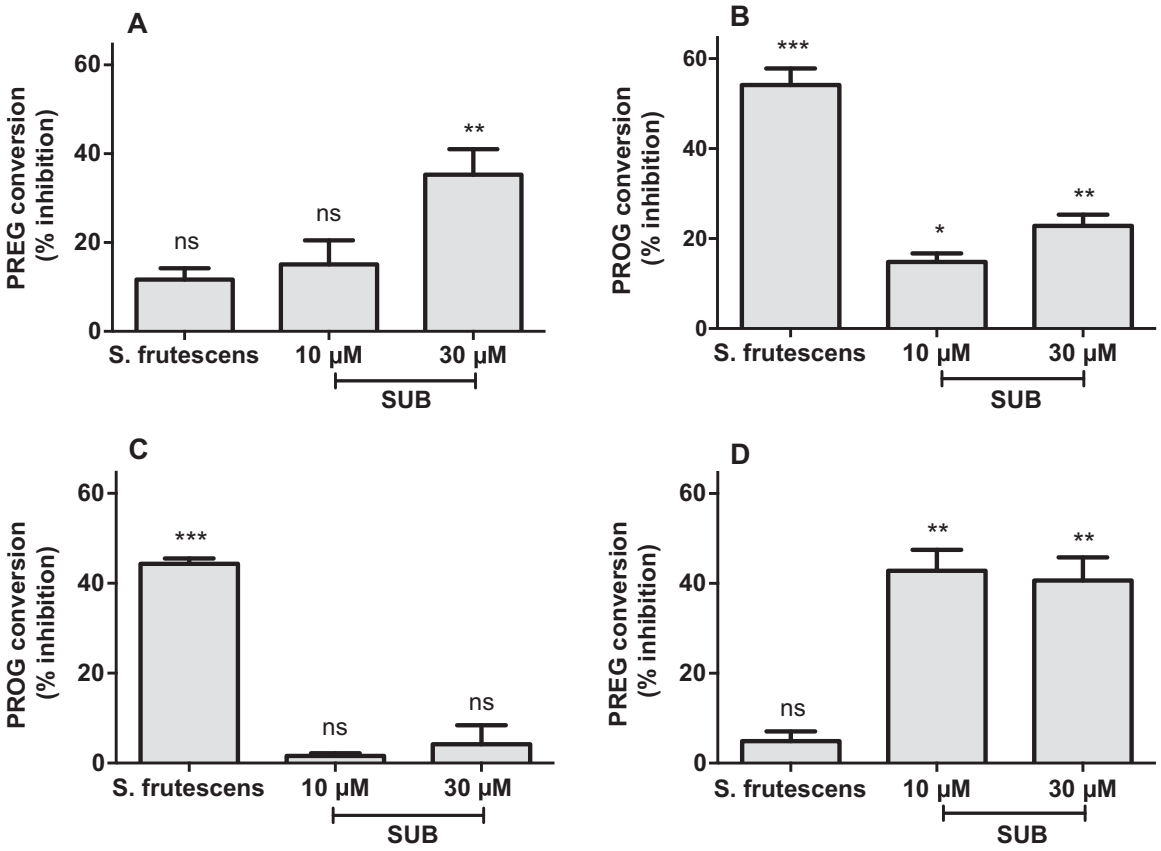

Fig. 4. Inhibition of substrate (1 µM) conversion by *S. frutescens* and SUB in transiently transfected COS-1 cells. Percentage inhibition of (A) PREG and (B) PROG conversion by CYP17A1; (C) PROG conversion by CYP21A2 and (D) PREG conversion by 3β-HSD2 in the presence of *S. frutescens* extract (2.6 mg/mL) and SUB (10 and 30 µM) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

2.7-fold, from 4 to 10.8 µM upon stimulation which, in the presence of extract, while not reaching basal levels, decreased significantly to 6.6 µM. All steroids in the three pathways decreased, and while only a few were not affected significantly, DHEAS levels in contrast increased significantly as in case of basal production.

3.4. *Influence of* S. frutescens *and SUB on GR and MR mediated gene transcription*

We subsequently investigated whether *S. frutescens* may elicit biological effects via the steroid receptor family, particularly the GR

Table 1
Steroids produced in H295R cells in the absence and presence of SUB after 48 h.

Steroid metabolite	Basal % of Total ± SEM	+10 µM SUB % of Total ± SEM	+30 µM SUB % of Total ± SEM
PREG	ND	ND	ND
PROG	0.00 ± 0.00	0.05 ± 0.00 ns	0.05 ± 0.00 ns
DOC	0.15 ± 0.00	0.20 ± 0.00 ns	0.30 ± 0.00 ns
CORT	6.30 ± 0.50	6.85 ± 1.15 ns	7.40 ± 1.55 ns
18OH-CORT	0.35 ± 0.10	0.45 ± 0.1 ns	0.50 ± 0.15 ns
ALDO	0.05 ± 0.05	0.05 ± 0.05 ns	0.15 ± 0.15 ns
11-DHC	0.70 ± 0.05	1.40 ± 0.30 ns	2.05 ± 0.55↑ $P < 0.05$
17OH-PREG	0.00 ± 0.00	0.00 ± 0.00 ns	0.00 ± 0.00 ns
17OH-PROG	1.15 ± 0.05	1.10 ± 0.05 ns	1.15 ± 0.05 ns
16OH-PROG	1.20 ± 0.00	1.10 ± 0.05 ns	0.90 ± 0.1↓ $P < 0.05$
Deoxycortisol	35.8 ± 1.75	39.30 ± 1.5 ns	40.60 ± 1.50 ns
Cortisol	23.10 ± 0.75	22.40 ± 0.4 ns	21.35 ± 0.80↓ $P < 0.05$
Cortisone	0.20 ± 0.05	0.15 ± 0.05 ns	0.15 ± 0.00 ns
DHEA	ND	0.48 ± 0.50 ns	ND
DHEAS	0.25 ± 0.00	0.14 ± 0.05 ns	0.15 ± 0.05 ns
A4	24.60 ± 0.50	21.1 ± 0.5↓ $P < 0.05$	21.00 ± 1.00↓ $P < 0.05$
11OH-A4	4.85 ± 0.50	4.25 ± 0.20↓ $P < 0.01$	3.40 ± 0.15$P < 0.001$
Testosterone	1.30 ± 0.05	1.05 ± 0.15 ns	0.90 ± 0.20 ns
Total steroid (nM)	4130.00 ± 133	3300.00 ± 650 ns	3200.00 ± 1235 ns

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Table 2
Steroids produced in H295R cells in the absence and presence of a methanolic extract of *S. frutescens* after 48 h.

Steroid metabolite	Basal	*S. frutescens* 1 mg/mL
	% of Total ± SEM	% of Total ± SEM
PREG	ND	ND
PROG	0.00 ± 0.00	0.00 ± 0.00 ns
DOC	0.15 ± 0.00	0.50 ± 0.05↑ P < 0.01
CORT	6.30 ± 0.60	4.30 ± 0.60↓ P < 0.001
18OH-CORT	0.35 ± 0.10	0.40 ± 0.10 ns
ALDO	0.05 ± 0.05	0.05 ± 0.00 ns
11-DHC	0.70 ± 0.05	1.00 ± 0.15 ns
17OH-PREG	0.00 ± 0.00	0.00 ± 0.00 ns
17OH-PROG	1.15 ± 0.05	1.65 ± 0.10 ns
16OH-PROG	1.20 ± 0.00	1.20 ± 0.05↓ P < 0.01
Deoxycortisol	35.80 ± 1.76	37.00 ± 1.10↓ P < 0.01
Cortisol	23.10 ± 0.75	17.60 ± 6.00↓ P < 0.001
Cortisone	0.2 ± 0.05	0.25 ± 0.05 ns
DHEA	ND	ND
DHEAS	0.25 ± 0.00	0.85 ± 0.15↑ P < 0.01
A4	24.60 ± 0.40	28.80 ± 0.70 ns
11OH-A4	4.85 ± 0.30	5.50 ± 0.50 ns
Testosterone	1.30 ± 0.5	1.25 ± 0.10 ns
Total steroid (nM)	4020.00 ± 90.00	2900.00 ± 135.5 ns

and MR. These receptors are ligand-dependent transcription factors that exhibit significant similarity in both their structures and mechanism of action. Like all steroid receptors, the GR and MR regulate gene expression by activating (transactivation) or repressing (transrepression) the transcription of target genes (Nicolaides et al., 2010; Nixon et al., 2013). Transactivation occurs when the ligand-activated GR or MR binds to GREs or mineralocorticoid response elements (MREs) in the promoters of specific genes, while transrepression occurs via protein-protein interactions with other transcription factors such as NF-κB, which drives the expression of many genes involved in the inflammatory response (Silverman and Sternberg, 2012).

The properties of the *S. frutescens* extract and SUB for transactivation of a synthetic GRE and transrepression of an IL-6 promoter-reporter gene were assayed in COS-1 cells. All assays were carried out using two *S. frutescens* concentrations, 0.5 and 0.75 mg/mL, and two SUB concentrations, 10 or 30 μM. For transactivation, the cells transiently expressing hGR and GRE-driven rat TAT-GRE-luciferase were treated with Dex (10 μM), the synthetic glucocorticoid, or with extract and SUB. Results show that, unlike Dex, neither the extract (Fig. 5A) nor SUB (Fig. 5B) display glucocorticoid agonist activity via the GRE at either concentration tested. For transrepression, COS-1 cells were transiently transfected with the hGR expression vector and the IL-6-NF-κB luciferase promoter reporter construct. After stimulation with 10 ng/mL PMA cells were exposed to Dex (10 μM), extract and SUB. The IL-6 promoter was significantly ($P < 0.001$) repressed by *S. frutescens* extract and SUB, with Dex displaying the greatest transrepression as expected (Fig. 6A and B).

Agonist and antagonist activity of *S. frutescens* towards the MR was subsequently investigated in CHO cells stably expressing the rat MR, 11β-HSD2 and a pTAT3-gLuc reporter gene (Morita et al., 1996). Cells were incubated with ALDO (10 nM), extract and SUB or 10 nM ALDO in the presence of extract and SUB, or spironolactone (1 μM). Neither the extract nor SUB displayed significant agonist activity for the MR at the concentrations assayed (Fig. 7A and B). However, comparable to spironolactone, the well-known MR antagonist, the effects of ALDO via the MR was antagonized by both the extract and SUB (Fig. 8A and B). Antagonism by SUB ($P < 0.05$) (Fig. 8B) was lower than that of the *S. frutescens* extract (Fig. 8A), while the latter antagonized the effect of ALDO comparable to that of spironolactone ($P < 0.001$).

4. Discussion

Traditional medicinal applications of *S. frutescens* are commonly used in the treatment of anxiety, stress and diabetes (Smith and Myburgh, 2004; van Wyk and Wink, 2004; Van Wyk, 2015), conditions linked to glucocorticoid dysfunction and inflammation, giving rise to our study into the effect of *S. frutescens* on adrenal hormone production and downstream receptor activation.

Since the triterpenoid SUs have structures similar to those of the steroid hormones it is plausible to assume that these compounds may interact with the P450 enzymes catalysing hormone biosynthesis. The P450 enzyme family, comprising of hepatic and steroidal P450 enzymes, are structurally conserved and studies suggest that herbal remedies that interact with hepatic P450 enzymes could interact with the steroidal P450 enzymes due to the structural homology of these enzymes. It has also been demonstrated that adverse events associated with herb-drug interactions may be attributed to the inhibition of hepatic P450 enzymes by bioactive compounds (Mrozikiewicz et al., 2010; Shi and Klotz, 2012; Zhou et al., 2003). Triterpenoid compounds in plants extracts have been linked to pharmacological effects which

Table 3
Steroids produced in forskolin-stimulated H295R cells in the absence and presence of a methanolic extract of *S. frutescens* after 48 h.

Steroid metabolite	10 μM Forskolin	10 μM Forskolin+*S. frutescens* 1 mg/mL
	% of Total ± SEM	% of Total ± SEM
PREG	ND	ND
PROG	0.00 ± 0.00	0.05 ± 0.00↓ P < 0.05
DOC	0.10 ± 0.00	0.15 ± 0.05↓ P < 0.05
CORT	16.75 ± 0.40	11.30 ± 0.30↓ P < 0.001
18OH-CORT	0.95 ± 0.05	1.00 ± 0.05 ns
ALDO	0.30 ± 0.10	0.30 ± 0.05 ns
11-DHC	0.55 ± 0.15	1.30 ± 0.25↓ P < 0.05
17OH-PREG	0.85 ± 0.20	1.35 ± 0.05↓ P < 0.05
17OH-PROG	0.25 ± 0.05	0.35 ± 0.00 ns
16OH-PROG	0.40 ± 0.00	0.50 ± 0.00↓ P < 0.01
Deoxycortisol	10.85 ± 2.00	20.70 ± 2.25↓ P < 0.01
Cortisol	41.55 ± 3.40	31.05 ± 2.05↓ P < 0.05
Cortisone	0.10 ± 0.00	0.05 ± 0.00 ns
DHEA	1.25 ± 0.45	1.95 ± 0.75 ns
DHEAS	0.10 ± 0.00	0.50 ± 0.10↑ P < 0.01
A4	10.25 ± 1.60	14.50 ± 0.80↓ P < 0.05
11OH-A4	8.90 ± 1.05	5.95 ± 0.60↓ P < 0.05
Testosterone	0.40 ± 0.05	0.55 ± 0.05 ns
Total steroid (nM)	10,790.00 ± 1765.00	6595.50 ± 555.00↓ P < 0.001

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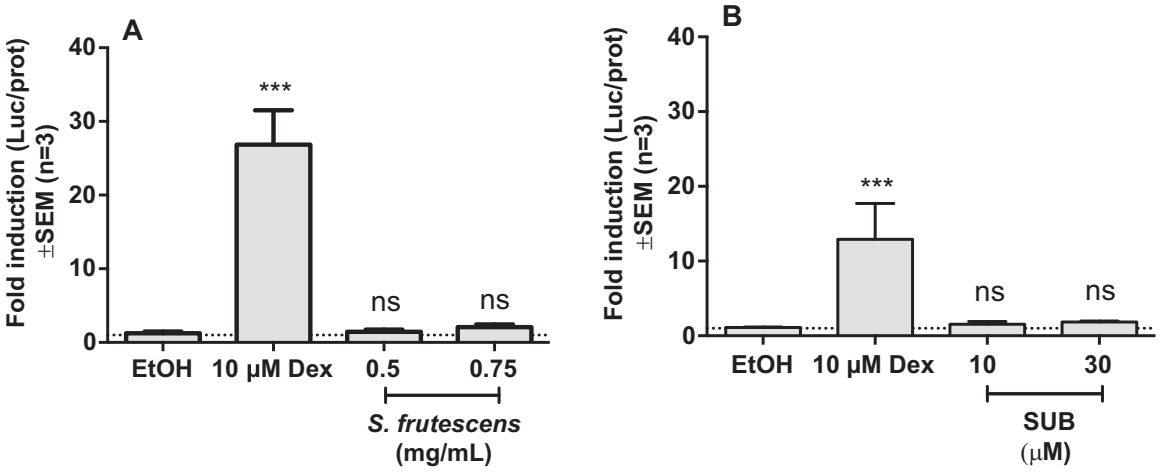

Fig. 5. *S. frutescens* and SUB exhibit no GR agonist activity for transactivation. Induction was assayed after 24 h in COS-1 cells transiently transfected with pRShGR and pTAT-GRE-E1b-luc reporter constructs exposed to vehicle (EtOH) and DEX (10 μM), (A) *S. frutescens* extract (0.5 and 0.75 mg/mL) and (B) SUB (10 and 30 μM). Results are shown as fold induction relative to EtOH set as 1 and results are representative of two experiments performed in triplicate. One-way ANOVA with Dunnett's posttest was used for statistical significance (***, $P < 0.001$; ns, non-significant).

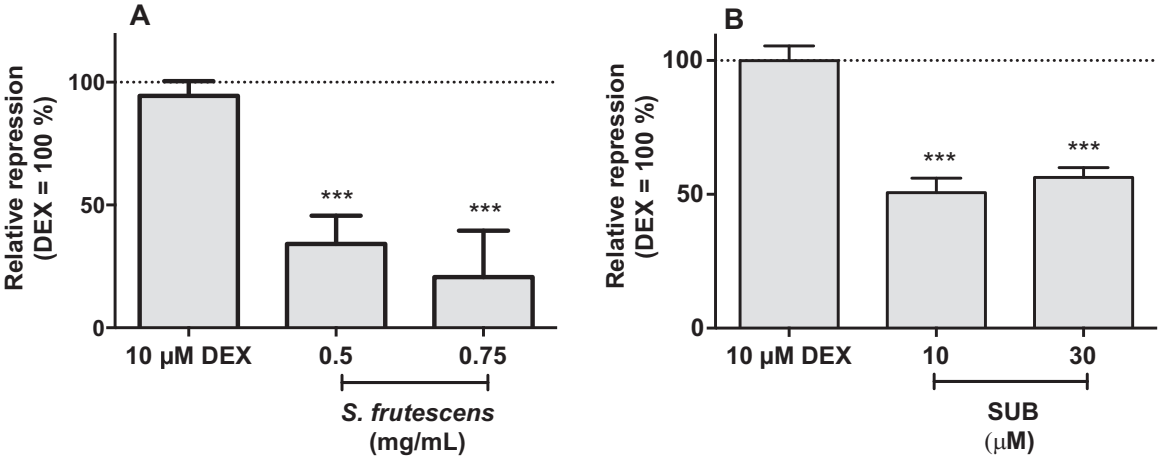

Fig. 6. *S. frutescens* and SUB exhibit GR agonist activity for transrepression. COS-1 cells transiently expressing the IL-6-NFκB-luc promoter-reporter construct were stimulated with PMA (10 ng/mL) and exposed to DEX (10 μM) and (A) *S. frutescens* extract (0.5 and 0.75 mg/mL) and (B) SUB (10 and 30 μM) for 24 h. Results are shown as the % repression of the extract and SUB expressed as a % relative to the DEX response set as 100%. Results are representative of two experiments each performed in triplicate. One-way ANOVA with Dunnett's posttest was used for statistical significance (***, $P < 0.001$; ns, non-significant).

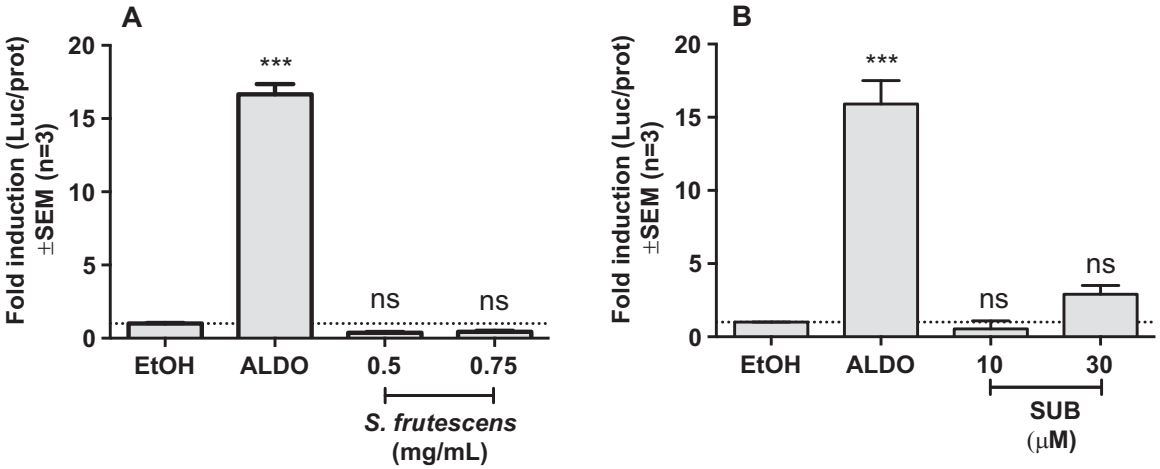

Fig. 7. *S. frutescens* and SUB exhibit no MR agonist activity for transactivation. CHO-11BHSD2 cells stably expressing rat MR and the pTAT3-gLuc reporter gene were incubated with vehicle (EtOH) and ALDO (10 nM) and (A) *S. frutescens* extract (0.5 and 0.75 mg/mL) and (B) SUB (10 and 30 μM) for 24 h. Results shown are representative of two experiments performed in triplicate. Fold induction relative to EtOH set as 1 is plotted. One-way ANOVA with Dunnett's posttest was used for statistical significance (***, $P < 0.001$; ns, non-significant).

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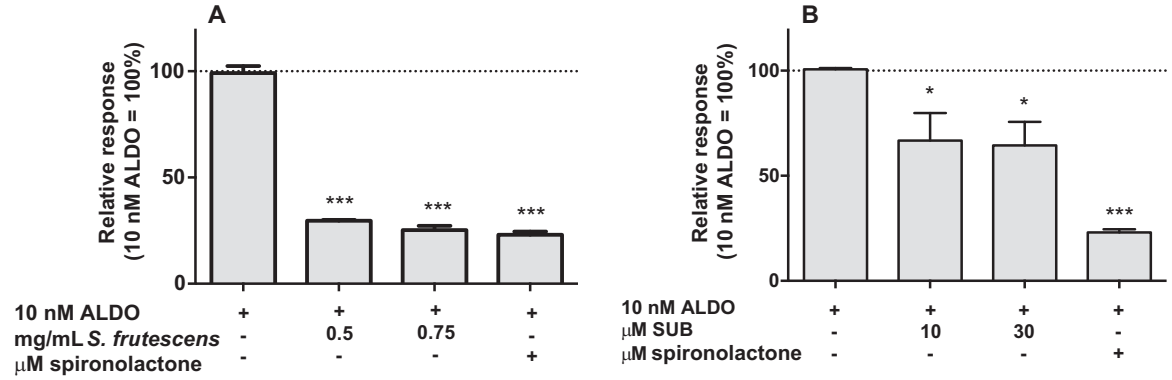

Fig. 8. *S. frutescens* and SUB exhibit MR antagonist activity for transactivation. CHO-11BHSD2 cells stably expressing rat MR and the pTAT3-gLuc reporter gene were incubated with ALDO (10 nM) set at 100% or (A) ALDO in the presence of *S. frutescens* extract (0.5 and 0.75 mg/mL) or spironolactone (1 μM), and (B) ALDO in the presence of SUB (10 and 30 μM) or spironolactone (1 μM) for 24 h. Results are representative of two experiments performed in triplicate (± SEM). One-way ANOVA with Dunnett's posttest was used for statistical significance (***, $P < 0.001$ ns, non-significant).

include the lowering of serum cholesterol levels as well as anti-inflammatory, cardiovascular and hypoglycemic effects (Desai et al., 2009; Lockyer et al., 2012; Wu et al., 2011). We have reported the modulation of adrenal P450 enzymes (Prevoo et al., 2004, 2008) with a more recent study reporting on *S. frutescens* extracts modulating P450 enzymes in human liver microsomal preparations, inhibiting the catalytic activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9 and CYP2C19 towards their known substrates in a dose dependent manner (Fasinu et al., 2013). In addition, it has been shown that while the ginsenosides present in ginseng are metabolized by hepatic P450 enzymes (Qi et al., 2011), they also inhibit these enzymes. Furthermore, the glycosyl moeiety of the compounds influences bioactivity, and has as such been shown to contribute to the resistance of ginsenoside to metabolism by CYP3A4 when compared to the metabolism of its aglycone. These data suggest that steric hindrances caused by the location of the glycosyl groups may prevent the ginsenosides from binding to the active pocket of CYP3A4 and from being metabolized (Hao et al., 2010). However, ginsenosides as well as the *S. frutescens* triterpenoids may bind sites other than the active site and may thus nevertheless hamper the catalytic activity of the P450 enzymes.

Although the SU compounds have similar cycloartane glycoside structures, they do differ markedly in their aglycone backbone specifically in terms of hydroxyl and ketone moieties at C1, C3 C7 and C11. SUA also has a C9/C11 double bond and an epoxy spanning C7/C10 over a 7-carbon ring structure while SUB, C and D have a methylene bridge between C9 and C10 (Avula et al., 2010; Fu et al., 2008). These structural differences permit these compounds to retain defined three dimensional structures which may contribute to different bioactive properties. In this study we turned our attention to SUB as the compound was present at concentrations marginally higher than the other SU compounds in the *S. frutescens* extract, with others having reported it to be the major SU in *S. frutescens* (Albrecht et al., 2012; Brownstein et al., 2015; Fu et al., 2010). While extraction methods would contribute to SU yields, studies have shown that secondary metabolites present in *S. frutescens* vary according to geographical location of plants, harvesting seasons and processing methods (Albrecht et al., 2012; Shaik et al., 2011). SUB specifically, has been shown to vary 0–1% (of dry mass) of *S. frutescens* plants grown in different geographical locations (Albrecht et al., 2012). Our results are in agreement with the expected range and the SUB yield constitutes 0.3% of the dry mass of *S. frutescens*.

We subsequently investigated the influence of a methanolic extract of *S. frutescens* and SUB on the catalytic activities of CYP17A1, CYP21A2 and 3β-HSD2 as these enzymes play a crucial role in adrenal steroidogenesis competing for the same substrates at pivotal branch points of the biosynthesis pathways with the modulation thereof potentially affecting downstream glucocorticoid, mineralocorticoid and androgen production. The inhibition of CYP17A1 would result in reduced cortisol and androgen production, whereas the inhibition of CYP21A2 would result in the inhibition of glucocorticoid and mineralocorticoid metabolites. Interference with 3β-HSD2 would reduce steroid metabolites in the three pathways. We previously showed that both aqueous and methanolic *S. frutescens* extracts hampered substrate binding to CYP17A1 and CYP21A1 and that the catalytic activities of CYP17A1 and CYP21A2 were inhibited by the aqueous extract. A mixture of triterpenoid compounds was also shown to inhibit both PREG and PROG binding to P450 enzymes. Furthermore, the triterpenoids were also able to bind to the P450 enzymes in the absence of the steroid substrate (Prevoo et al., 2008). These data clearly indicated that *S. frutescens* interacted and modulated the activity of the steroidogenic P450 enzymes prompting further investigations. In the present study we used the methanolic extract of *S. frutescens* to investigate inhibitory effects and showed the inhibition of *S. frutescens* on CYP17A1 and CYP21A2 in the conversion of PROG, with the inhibition of PREG conversion by CYP17A1 being significantly lower. Taken together, these data suggest that the extract would affect the production of adrenal steroids. The present study also showed that in the presence of SUB only, the catalytic activity of CYP17A1 towards both substrates was inhibited together with 3β-HSD2 suggesting that the compound would affect steroidogenesis at the branch point, albeit somewhat differently when compared to the extract, modulating the flux in all steroidogenic pathways and affecting total steroid production. The data suggests that inhibition at the branch point and subsequent changes in steroid output may, in part, therefore be attributed to the SU compounds.

Downstream effects of *S. frutescens* on steroid hormone production in the H296R cell model showed this to be the case. Basal steroid production was similar to that previously reported (Mangelis et al., 2016; Rege et al., 2013; Schloms et al., 2012; Xing et al., 2011) and while basal steroid production decreased in the presence of SUB (Table 1), data showed a decreased steroid shunt in the androgen pathway (1.5-fold) as well as in the glucocorticoid pathway (1.2-fold) suggesting inhibition of CYP17A1. CYP17A1's inhibition was also reflected in the 1.75-fold decrease in 16OH-PROG levels, a steroid not metabolized further in the adrenal. The data also suggests CYP11B1 inhibition with the significantly decreased cortisol (1.4-fold) and 11OHA4 (1.8-fold) levels. In addition, even though A4 production was significantly reduced, testosterone production did not change significantly. Although the adrenal is not the primary site of testosterone production, the production of this hormone is of importance in females (Goymann and Wingfield, 2014). 11-DHC production increased significantly indicative of the inactivation of CORT by 11β-HSD2 which is expressed at low levels in the adrenal (Rege et al.,

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2013). It is interesting to note that under basal conditions SUB increased 11-DHC levels (2.3-fold) at the higher concentration.

Although both CYP17A1 and 3β-HSD2 were inhibited by SUB when expressed in isolation, these modulatory effects are not always obvious in a complex model system with endogenous expression of steroidogenic enzymes. Steroid conversion would, in the H295R model system, also be dependent on competition between enzymes for the same substrates, together with the availability of precursors. However, analyses clearly indicates inhibition of CYP17A1 by SUB which would impact on the availability of substrates for the downstream conversion by 3β-HSD2 and CYP21A2. In contrast, in the presence of *S. frutescens* extract, steroid flux in the three pathways was affected. Steroid metabolites in the glucocorticoid pathway decreased 1.5-fold and 1.2-fold in the androgen pathway. It would seem that Δ5-steroids channelled towards DHEA were sulphonated efficiently, reflected in the significantly increased DHEAS levels (2.66-fold), resulting in changes in downstream adrenal androgens levels and perhaps indicating stimulation of sulfotransferase (SULT2A1). In contrast to SUB, the extract modulated the mineralocorticoid pathway resulting in a 1.7-fold decrease in steroid metabolites. The flux through the glucocorticoid pathway decreased 1.5-fold, comparable to SUB, resulting in a 1.8-fold decrease in cortisol levels as well as a significant decrease in the levels of the precursor steroid, deoxycortisol. Interestingly the significant reduction in CORT production (2-fold) strongly suggests downstream inhibition of CYP11B1/2 which would contribute to the increased DOC levels. The increased shunt towards the production of inactive glucocorticoids we observed in our H295R assays may indicate a modulation of 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) activity. The data furthermore also suggests a stimulatory activity for *S. frutescens* towards SULT2A1, which would contribute towards the inactivation of adrenal androgens with potential clinical applications in disease conditions which are charcarterized by excess adrenal androgens. Taken together, the catalytic activity of *S. frutescens* towards these two enzymes warrant further investigations as these are closely associated with metabolic diseases and clinical conditions.

Our data showed a significant reduction of CORT production observed under stress-mimicing conditions in the adrenal cell model (Table 3) together with an *in vivo* study by Smith and Myburg 2004, also showing that the intraperitonial administration of *S. frutescens* extracts to male Wistar rats resulted in decreased CORT plasma levels ($P < 0.05$) when rats were exposed to chronic immobilization stress (Smith and Myburgh, 2004). It should be noted that CORT is the major glucocorticoid in rats since CYP17A1 is not expressed in the rat adrenal and thus cortisol is not produced. In the same study the authors also reported that the administration of the extract resulted in decreased IL-6 levels highlighting the anti-inflammatory properties of *S. frutescens* in lowering the pro-inflammatory cytokine level under basal conditions. However, in a subsequent study by Smith and van Vuuren (2014), investigating central mechanisms of action after a single *S. frutescens* dose was given by gavage, basal CORT levels did not change significantly compared to the placebo group and after acute immobilization stress levels did not differ significantly from the placebo group. However, CORT was significantly increased in both groups, which was attributed to hormone levels being assayed immediately after exposure to the stress intervention. Interestingly, adding to *S. frutescens*'s anti-inflammatory properties is their observation that the stress-induced down-regulation of the GR was abolished by the ingestion of the *S. frutescens* extract.

The inhibitory effects of the extract on CYP17A1 were apparent in the significantly reduced levels of 16OH-PROG (1.4-fold). It should be noted that SUB, at 10 μM and 30 μM inhibited 16OH-PROG production 1.36- and 1.75-fold, respectively, while the concentration of SUB in the extract was ± 47 μM. The other SU compounds (A, C and D) where present in the extract at ± 100 μM (collectively) and together with the sutherlandin flavonoid compounds would contribute to the modulation of steroidogenesis. In addition to the sutherlandins, rutin, kaaempferol-3-o-rutinoside, quercitrin, quercetin and kaempferol have also been identified within *S. frutescens* extracts (Mbamalu et al., 2016). We have shown that flavonoid compounds, specifically rutin, inhibited specific steroidogenic enzymes affecting the outcome of steroidogenesis in the H295R model (Schloms et al., 2012; Schloms and Swart, 2014), affects we also showed in *in vivo* studies. It should be noted that while it is possible that these compounds may have different effects on different enzymes, either inhibitory or stimulatory, they may, being present at such high concentrations, aggregate when the extract is assayed under experimental conditions thus diminishing their influence.

Stimulation of the H295R cells with forskolin resulted in a 2.6-fold increase of total steroid production and while steroid metabolites increased in the mineralocorticoid and glucocorticoid pathways and to a lesser degree in the androgen pathway, stimulation was attenuated significantly, 2-, 1.6- and 1.5-fold, respectively, by the extract. DHEAS levels were again increased significantly even though CYP17A1 was inhibited in the presence of the extract which resulted in a decreased production of downstream androgens. CORT and cortisol production were decreased 2.4- and 2.2-fold, respectively, while their precursor steroid levels were significantly increased, suggesting inhibition of CYP11B1.

The data obtained indicate that the presence of *S. frutescens* extract influenced glucocorticoid and mineralocorticoid production to a greater extent. The inhibition of DOC conversion appeared to be consistent in the presence of the extract under stimulated and basal conditions. DOC plays an integral role in glucocorticoid and mineralocorticoid production as this steroid substrate can either be converted to CORT (a glucocorticoid) by CYP11B1 or to ALDO (the major mineralocorticoid) by CYP11B2. In addition to acting like a glucocorticoid in many functions such as the stress response, DOC has also been shown to act as a significant mineralocorticoid in downstream signalling events (Vinson, 2011). Both DOC and deoxycortisol have been shown to play a significant role in hypertension and hyperkalemia as both these glucocorticoids can act as mineralocorticoids through binding to the MR (Sugimoto et al., 2016).

Given the data demonstrating the reduction of CORT and cortisol, as well as that of the steroid metabolites in the mineralocorticoid pathway, both under basal and stimulated conditions, we subsequently investigated the effect of the extract on the GR and the MR to determine downstream cellular effects. Exogenous glucocorticoids acting via the GR are widely used to treat inflammatory disorders. The clinical usage of glucocorticoids is however limited, due to multiple adverse side-effects associated with prolonged use which include, amongst others, mood and behavioural changes, adiposity and steroid-induced diabetes (Whitehouse, 2011). These side-effects are generally reported to be due to their capacity to transactivate gene transcription (Baschant et al., 2013) while the therapeutic anti-inflammatory properties are generally considered to be due to the transrepression mechanism (Nixon et al., 2013). The latter concept has however been called into question recently as it was found that the negative side-effects associated with anti-inflammatory treatments may in fact result from both transactivation as well as transrepression mechanisms of the GR (Coutinho and Chapman, 2011; Sundahl et al., 2015). However, potential anti-inflammatory compounds with fewer side-effects which stimulate and ultimately modify GR-mediated gene expression profiles, are still actively being sought (Vandevyver et al., 2013). These compounds are referred to as dissociated compounds or selective glucocorticoid receptor agonists (SEGRAs). One of the first SEGRAs to be described was Compound A, a non-steroidal compound displaying no transactivation of GRE-mediated gene transcription while repressing NF-κB-driven gene expression (De Bosscher et al., 2005; Gossye et al., 2010). Collectively, our transactivation and transrepression results show that the *S. frutescens* extract and SUB can efficiently repress NF-κB-driven gene expression, without activating GRE-driven gene expression suggesting that the plant-derived SUB

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is a SEGRA which may have anti-inflammatory applications.

In addition to regulating the inflammatory response, circulating glucocorticoids have also been found to induce hypertension. Although it was originally thought that essential hypertension was mediated by sodium imbalances, recent data have suggested that the pathogenesis and maintenance of glucocorticoid-induced hypertension is mediated by the activation of the mineralocorticoid receptor (MR) by glucocorticoids (Whitworth et al., 2000). Typical hypertension treatments include the administration of MR antagonists such as spironolactone. Studies have furthermore shown a strong association between inflammation and congestive heart failure. Although the release of pro-inflammatory cytokines are major contributing factors to the onset of inflammation-induced congestive heart failure (Dick and Epelman, 2016), glucocorticoids, the GR and the MR have also been shown to play significant roles (Fuller, 2015; Messaoudi et al., 2012). Although the circulating glucocorticoid concentrations are significantly higher compared to ALDO (± 1000-fold), the binding of glucocorticoids to the MR is regulated by the presence of 11βHSD2. 11βHSD2 converts cortisol and CORT to their inactive forms, cortisone and 11-DHC respectively which have no affinity for the MR (Edwards, 1988).

Our results showed that the *S. frutescens* extract and SUB did not activate MR mediated gene transcription, but instead antagonized the effects of ALDO via the MR. This result strongly suggests that the extract and SUB could be used as in anti-hypertensive treatment. The finding that *S. frutescens* also contain cardenolides, naturally occurring cardiac glycosides, also suggest a potential application as a herbal remedy in the treatment of congestive heart failure in addition to its traditional use in treating inflammation (Shaik et al., 2011). The findings that *S. frutescens* acts as a SERGA is novel, however, further studies are required to show, beyond doubt, that the medicinal plant acts as a dissociating GR ligand, preventing GR dimerization and whether GR transrepression would also be detected in vivo. In addition, investigations into the activation of downstream GR target genes involved in anti-inflammatory responses would further contribute towards elucidating the SERGA properties of *S. frutescens*.

The specific inhibition of adrenal steroidogenic enzymes by *S. frutescens* at the branch point in steroidogenesis modulated hormone production in the mineralocorticoid, glucocorticoid and androgens pathways. Decreased glucocorticoid production together with the repression of NF-κB-driven gene expression without GRE-driven gene activation by *S. frutescens* unequivocally links the anti-stress and the anti-inflammatory bioactivities of the medicinal plant to adrenal hormone production and receptor interaction. In addition, the inhibitory effects observed in the mineralocorticoid pathway together with the antagonistic effects of *S. frutescens* on ALDO's activation of the MR have furthermore revealed a novel facet in the plant's bioactivity in terms of anti-hypertensive effects.

5. Conclusion

The outcome of our steroidogenic enzyme assays show that *S. frutescens* extracts affect the catalytic activities of CYP17A1 and CYP21A2 while not influencing 3β-HSD2. On the other hand, SUB one of the major triterpenoids in *S. frutescens*, inhibited both CYP17A1 and 3β-HSD2 and would thus perturb steroidogenesis at the branch point. Regarding the outcome in the human H295R adrenal cell model it is clear that the extract and SUB modulate the flux in the steroidogenic pathways. However, the effects of *S. frutescens* are most prominent under conditions mimicking the stress response in which the extract reduced steroid production in the three pathways with the greatest modulation observed in the mineralocorticoid pathway. There was a significant reduction in glucocorticoid production (CORT and cortisol) and an increased shunt towards DHEAS, contributing to the decreased production of androgen precursors. These data confirm the inhibition of CYP17A1 while also suggesting that *S. frutescens* may inhibit both CYP11B isoforms, which may possibly be attributed to the SU and sutherlandin compounds being present at far higher concentrations than the 30 μM SUB assayed in this study. However, at this concentration cortisol and 11OHA4 production was inhibited significantly. These effects may contribute to the stress relieving and anti-anxiety bioactivities of *S. frutescens* and to the potential anti-hypertensive effects. It has been suggested that *S. frutescens* elicits adaptogen-like effects, favouring the body's ability to adapt to stressors, increasing resistance to stress and disease by maintaining functional CORT levels following a study showing that the medicinal plant decreased CORT levels in rats subjected to chronic stress (Smith and Myburgh, 2004). Our findings, together with the anti-inflammatory and corticomimetic reports of triterpenoids (van Wyk and Wink, 2004) and the fact that it is generally accepted that inflammatory responses are associated with the pathiophysiology of hypertension and the development of cardiovascular disease prompted our investigation into the effects on GR and MR driven gene transcription. This led to one of the most important findings of our study - *S. frutescens* extracts and SUB act like SEGRAs by selectively triggering transrepression and not transactivation. Regarding the GR, *S. frutescens* and SUB exhibited no transactivation potential on GRE-driven gene expression, and repressed NF-κB-driven gene expression, while both exhibited MR antagonist activity for transactivation. Although further studies are required to determine the *in vivo* effects, data suggest that *S. frutescens* is a promising candidate in anti-inflammatory therapeutics with reduced side-effects as well as in anti-hypertensive strategies. It is also possible that the anti-inflammatory and anti-hypertensive properties of *S. frutescens* may be attributed to further downstream transcriptional effects via the GR and MR. These results should however not be viewed in isolation as the complex pathophysiology of hypertension and interactions between antagonists and the MR in cardiovascular disease remains uncertain. Notwithstanding, our study has, for the first time, provided a scientific base for anti-inflammatory effects reflected in *S. frutescens's* efficient repression of NF-κB-driven gene expression without GRE-driven gene activation, and anti-hypertensive properties in which *S. frutescens* was unable to activate MR mediated gene transcription while antagonising ALDO's MR effects.

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