The influence of *Sutherlandia frutescens* on adrenal cytochrome P450 11β-hydroxylase

by

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Declaration

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Summary

This study:

1. describes the preparation of a methanol extract of *Sutherlandia frutescens* and the HPLC fractionation of the methanol extract.

2. investigates the influence of *S. frutescens* on the binding properties of mitochondrial cytochrome 11β-hydroxylase (CYP11B1) to deoxycorticosterone (DOC) and deoxycortisol, demonstrating that methanol extracts of *S. frutescens* inhibit the Type I substrate-induced difference spectra.

3. investigates the influence of *S. frutescens* on the catalytic activity of CYP11B1 expressed in COS1 cells, demonstrating that the methanol extract of *S. frutescens* inhibits the conversion of DOC and deoxycortisol.

4. describes the sequential extraction of the methanol extract of *S. frutescens* using organic solvents and the inhibition of the conversion of DOC by CYP11B1 expressed in COS1 cells in the presence of these extracts.

5. describes the inhibition of the binding of DOC to CYP11B1 in ovine adrenal mitochondria, and the conversion of DOC by CYP11B1 expressed in COS1 cells by these fractions.

6. identifies the presence of the flavonoid compounds, orientin vitexin and rutin, in *S. frutescens.*

7. investigates the influence of the flavonoid compounds on the binding of DOC to CYP11B1 and on the catalytic activity of DOC by CYP11B1 expressed in COS1 cells.

8. identifies the presence of the triterpenoid, sutherlandioside A (SU1), in *S. frutescens* extracts and investigates its effect on the binding of DOC to CYP11B1.

Opsomming

Hierdie studie beskryf:

1. die voorbereiding van ‘n metanol ekstraksie van *Sutherlandia frutescens* en die HPLC fraksionering van die metanol ekstrakte.

2. ‘n ondersoek na die invloed van *S. frutescens* op die bindingseienskappe van sitochroom P450 11β-hidroksilase (CYP11B1) in skaap bynier mitochondria en demonstreer dat *S. frutescens* metanol ekstrakte die vorming van steroïed-geinduseerde tipe I verskil spektra van deoksiekortisol en deoksikortikosteroon (DOC) inhibeer.

3. ‘n ondersoek na die invloed van *S. frutescens* op die katalitiese aktiwiteit van CYP11B1 in COS1 selle en demonstreer die inhibisie van DOC en deoksikortisol omsetting na hul produkte deur die methanol ekstrakte.

4. die opeenvolgende ekstraksie van methanol extrakte van *S. frutescens* met organiese oplosmiddels en beskryf die inhibisie van die CYP11B1 gekataliseerde omsetting van DOC in COS1selle in die teenwoordigheid van die ekstrakte.

5. die inhibeerende effek op die binding van DOC aan CYP11B1 in skaap bynier mitochondria en die inhibisie van die CYP11B1 gekataliseerde omsetting van DOC in COS1selle.

6. die identifisering van flavonoïed verbindings, orientin vitexin en rutin in *S. frutescens.*

7. ‘n ondersoek na die invloed van die flavonoïed verbindings op die binding van DOC aan CYP11B1 en op die katalitiese aktiwiteit van CYP11B1 in COS1 selle.

8. die indentifisering van die triterpenoïed, sutherlandiosied A (SU1), in *S. frutescens* en ondersoek die invloed van SU1 op die binding van DOC aan CYP11B1.

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Table of Contents

Chapter 4

Chapter 5

Abbreviations

11β HSD	11β-hydroxysteroid dehydrogenase
8-OHdG	8-hydroxy-2'-deoxyguanosine
3βHSD	3β-hydroxysteroid dehydrogenase
A4	androstenedione
ACTH	adrenocorticotropic hormone
ADX	adrenodoxin
ADXR	adrenodoxin reductase
AF-1	activation function-1
ANS	autonomic nervous system
AP-1	activator protein-1
APCI	atmospheric pressure chemical ionization
AVP	arginine vasopressin
BSA	bovine serum albumin
cAMP	adenosine 3'5'-cyclic monophosphate
CBG	cortisol binding globulin
CNS	central nervous system
COS1	cells transformed African green monkey tumor cells
COX-2	cyclooxygenase-2
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
CRH	corticotrophin releasing hormone
CYP11A	cytochrome P450 cholesterol side chain cleavage
CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17	cytochrome P450 17 β-hydroxylase
CYP21	cytochrome P450 21-hydroxylase
DAD	diode array detection
DHAS	dehydroepiandrosterone sulphate
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's Modified Eagles Medium

DMSO	dimethylsulphoxide
DOC	11-deoxycorticosterone
E	epinephrine
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal regulated protein kinase
ESMS	electron spray mass spectrometry
FCS	fetal calf serum
FFA	free fatty acid
FSH	follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GH	growth hormone
GnRH	gonadotrophin-releasing hormone
GR	glucocorticoid receptor
HCN	hydroprussic acid
HDL	high density lipoproteins
HIV	Human Immunodeficiency Virus
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
HSP	heat shock proteins
IGF	insulin-like growth factor
IL-1β	interleukin-1β
IP_3	inisitol triphosphate
LCMS	liquid chromatography mass spectrometry
LDL	low density lipoproteins
LH	luteinizing hormone
MC	microcallus
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MR	mineralocorticoid receptor
MT	missing trabecular structure

NADP(H)	nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
NF-κB	Nuclear factor- kappa B
NIH NCAM	National Institutes of Health and National Centre for Complementary and Alternative Medicine
NO	Nitric oxide
NPAA(s)	non-protein amino acid(s)
NRF	National Research Foundation
NSAIDs	nonsteroidal anti-inflammatory drugs
OB	osteoblasts
OC	osteoclasts
PEG	poly ethylene glycol
POMC	proopiomelanocortin
PTH	parathyroid hormone
PTH	perforated trabecular structure
PVN	paraventricular nucleus
ROR	reactive oxygen radical
SCN	suprachiasmatic nucleus
SF-1	steroidogenic factor-1
SNS	sympatic nervous system
SP	Substance P
SRS	substrate recognition sequences
StAR	steroidogenic acute regulatory protein
THE	tetrahydrocortisone
THF	tetrahydrocortisol
TNF-α	tumor necrosis factor- alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TSH	thyroid-stimulating hormone
UV	ultra violet

Chapter 1

Introduction

Southern Africa has a rich floral diversity which has been exploited for traditional medicinal practices. Traditional medicines are culturally significant in Southern Africa, India and China and constitute the primary healthcare system of approximately 27 million South Africans [Mander (1998)]. Despite the fact that these practices and medicines are thought to be primitive, their popularity in first world countries has increased over the last decade as a result of consumers shying away from orthodox medicines [Barnes *et al* (2002)]. However, a major concern regarding the use of traditional medicines is the lack of scientific data regarding safety and efficacy since therapeutic claims associated with the use of traditional medicines are mainly anecdotal. Although the recently renewed interest in medicinal plants has encouraged researchers to scientifically validate the use of these plants in treatments, it has also promoted the discovery of compounds with pharmaceutical value [Van Wyk (2002)].

Although the safety and efficacy of traditional medicines and the compounds within these medicines are of scientific interest, the unregulated prescription and use of these medicines is a major health concern. Traditional healers play important roles in distribution, prescription and harvesting of medicinal plants and all these factors affect the final product used by the consumer. The issues surrounding the use of traditional medicines and the practitioners that provide these medicines are discussed in Chapter 2.

Sutherlandia frutescens is a medicinal plant that has been used extensively in traditional medicinal practices [Smith (1895); Watt & Breyer-Brandewijk (1962)]. Decoctions made from this plant have been used to treat a wide variety of ailments such as diabetes, internal cancers, stomach problems, inflammation, anxiety, depression and stress. The ability of *S. frutescens* to treat these ailments has been attributed to the presence of various bioactive compounds, of which canavanine, pinitol, gamma aminobutyric acid (GABA) and cycloartane glycosides, sutherlandioside A, B, C and D, have been identified [Van Wyk *et al* (2000); Van Wyk *et al* (2008); Van Wyk & Albrecht (2008)]. Although therapeutic claims associated with the use of *S. frutescens* have been mainly

anecdotal, the claim that this medicinal plant is an effective treatment for cancer and the Human Immunodeficiency Virus/Autoimmune Deficiency Syndrome (HIV/AIDS) has raised scientific interest in this plant. Subsequent investigations have led to the accumulation of mostly pre-clinical data with only a few clinical studies being conducted [Van Wyk (2002); Van Wyk & Albrecht (2008)]. The properties of *S. frutescens*, the compounds found within this medicinal plant and its application in the treatment of the abovementioned ailments are discussed in Chapter 3.

Bioactive compounds in medicinal plants are metabolized in the liver by cytochrome P450 (P450) enzymes. These enzymes mediate the phase I oxidative metabolism of approximately 90 % of all known drugs, of which approximately 69 % are metabolized by CYP3A4, CYP2D6 and CYP2C [Lewis (2003)]. It is not uncommon for drugs to inhibit the metabolism of compounds by the P450 enzymes, often resulting in the accumulation of drugs in the body, at times to a toxic extent. An example of such an interaction is grapefruit juice and cardiovascular medication. Compounds found in grapefruit juice inhibit the function of CYP3A4, impairing the body's ability to metabolize foreign compounds to less harmful compounds. This leads to the accumulation of the drug and a strong possibility of overdosing [Bailey and Dresser (2004)]. Although hepatic P450 enzymes are responsible for the metabolism of drugs and foreign compounds, P450 enzymes are expressed in many other tissues and play major roles in steroid metabolism, cholesterol synthesis and vitamin D metabolism [Payne & Hales (2004)]. Since the P450 superfamily is highly homologous, it is possible that xenobiotics not only interact with hepatic enzymes but also with the steroidogenic enzymes. These compounds may affect the steroidogenic enzymes, resulting in the altered production of androgens, mineralocorticoids and glucocorticoids [Porterfield (2001)].

The biosynthesis of adrenal steroid hormones is strictly regulated within the human endocrine system. The enzymes responsible for the biosynthesis of the adrenal steroid hormones, including cortisol, are the adrenal steroidogenic P450 enzymes. When the human body is exposed to a stress stimulus, the hypothalamic-pituitary-adrenal (HPA)-axis is activated. The activation of this cascade of reactions ultimately results in elevated

plasma glucocorticoid levels, specifically cortisol. In a healthy individual, the elevated cortisol levels induce a negative feedback mechanism which inhibits the reactions associated with the activation of the HPA-axis, consequently allowing cortisol levels to return to normal. In some cases, as with chronic stress, the stress stimulus persists for a prolonged period, desensitizing the activation of the HPA axis [Jacobson (2005)]. This results in permanently elevated cortisol levels. The long-term exposure to cortisol has various adverse effects which include osteoporosis, type 2 diabetes, impairment of the immune and central nervous system, many of which have been linked to dysfunctional steroidogenesis [Chrousos *et al* (1998); Leverenz *et al* (1999)]. Adrenal steroidogenesis and the influence of P450 enzymes on the production of cortisol are discussed in Chapter 4. This chapter also includes a discussion on the adverse effects associated with elevated plasma cortisol levels.

Elevated cortisol levels could be treated by inhibiting the production of cortisol, possibly by inhibiting the steroidogenic P450 enzymes. The influence of *S. frutescens* and compounds in this medicinal plant on the catalytic activity of cytochrome P450 11β-hydroxylase (CYP11B1), the enzyme which catalyses the final step in cortisol biosynthesis is presented in Chapter 5. During this study several extracts and fractionation methods were tested for bioactivity by investigating their ability to influence substrate binding to CYP11B1 as well as substrate conversion by the enzyme. Due to the unique spectral properties of cytochrome P450 enzymes, the inhibition of deoxycorticosterone (DOC) and deoxycortisol binding to ovine adrenal mitochondrial CYP11B1 could be monitored spectrophotometrically. The ability of *S. frutescens* to inhibit the conversion of DOC and deoxycortisol was investigated in COS1 cells expressing baboon CYP11B1. In an attempt to identify the compounds responsible for the bioactivity of *S. frutescens*, the extracts were fractionated and subjected to liquid chromatography mass spectrometry (LC-MS). Flavonoid compounds were identified and their bioactivity subsequently investigated.

Chapter 6 is a summary of the results obtained in this study and the conclusions drawn from these results.

Chapter 2

Traditional medicines in South Africa

Traditional medicines play an integral role in many cultures in South Africa. It is estimated that approximately 27 million people in South Africa currently rely on traditional medicines for their primary healthcare [Mander *et al* (1996)]. Within this sector traditional healers act as "medical practitioners" with many people dependent on their advice and the products they provide. There are, however, flaws within the system although several laws have been passed to regulate healers and practices for the safety of the consumers. In addition, various studies have been conducted to determine the safety and efficacy of traditional medicines [Schimmer *et al* (1988); Johnston *et al* (2007)]. Compounds have been identified in traditional medicines and have been linked to diverse pharmacological effects. Although various studies have been implemented to address the concerns surrounding the safety and toxicology of identified compounds, the accumulation of preclinical data outweighs the clinical data presently available. This Chapter focuses on aspects of the traditional medicine practice and trade that are often overlooked such as the role of traditional healers and ethnopharmacological research associated with safety and efficacy of whole plant extracts and the compounds identified within these extracts.

2.1 The role of traditional healers

South Africa is rich in floral diversity with nearly 30 000 species of higher plants prevalent to the region [Goldblatt (1978)]. Africans take advantage of this diversity using several plants in herbal remedies and traditional medicines. Traditional medicines have origins all over the world with one of the oldest and most diverse traditional medicines being from the native tribes of the African continent, especially those associated with the San and the Khoi [Van Wyk & Wink (2004)]. Unfortunately, many of these medicinal systems are poorly recorded. Within these cultures, traditional medicines are usually recommended and distributed by traditional healers known by the natives as "inyanga" and "isangoma". The medicinal plant tonics and infusions recommended by these healers are said to sustain general physical health in healthy individuals. In the case of disease,

these medicines are said to aid the body in self regeneration and possibly also aid the immune system [Van Wyk *et al* (1997); Van Wyk *et al* (2000); Van Wyk & Wink (2004)].

In South Africa, traditional medicines are more accessible than orthodox medicines and form an integral part of many cultures. Traditional medicines made from plant extracts are categorized as phytomedicines. Although it is generally accepted that phytomedicines are standardized and quantified, this is not applicable to traditional medicines. As in many developing countries, health care in rural South Africa depends largely on traditional medicines. It is thought that up to 70 % of the population still relies on traditional medicines to provide primary health care. Although cultural importance is one of the reasons for the high demand for traditional medicines, the main contributing factor is still the limited availability and high cost of orthodox medicines [McGaw *et al* (2005)]. In addition, a change of government has contributed to an increased awareness and demand for these medicines and has also led to the development of many herbalist organizations [Devenish (2005)]. Previous legislation, such as the Medicines and Regulated Substance control act (Act 101 of 1965), set several guidelines and regulations for traditional medicines which were, however, not regulated or enforced. Subsequently, legislation was passed (Health Act of 1974) which banned traditional healers. These practices fortunately continued with access to traditional medicines subsequently becoming a priority for the national government. Traditional healers are now recognized within the health care system [Digby (2005)]. The Health Practitioners Act (2004) sets regulations for the training, conduct and registration of traditional healers as well as regulations concerning quality, efficacy and safety of the services provided by the traditional healers. The recognition of these traditional healers resulted in a higher demand for medicinal plants, leading to illegal harvesting with some species nearing extinction. [Zschocke *et* al (2000); Medical Research Council (2005)]. In addition, traditional healers tend to believe that the whole plant extract is a more potent remedy than single compounds isolated from these extracts [Rodriguez-Fragoso *et al* (2008)]. This may influence propagation and seeding since whole plants are uprooted, leading to a rapid decrease of specie numbers and ultimately extinction. Various communities have

subsequently taken on conservation projects contributing to the stabilization of the delicate status of medicinal plants in the wild. In addition, their aim is to promote strong social and cultural concerns while generating sustainable projects [Wiersum *et al* (2006)].

As with the majority of unregulated practices, the practices of traditional healers are not without flaws. The most common problems that occur usually involve concurrent use of traditional and orthodox healthcare systems. In many cases patients do not disclose their use of traditional medicines to medical doctors or forensic experts [Stewart & Steenkamp (2000)] since these individuals may regard the use of traditional medicines as primitive. In addition, orthodox pharmaceuticals often have adverse effects when taken in conjunction with herbal remedies often leading to poisoning [Izzo & Ernst (2001)]. The concurrent use of different traditional medicines may also lead to synergistic effects, some being mutagenic and/or cytotoxic [Barnes (2003); Fennell *et al* (2004b)].

The incidence of untrained and unregulated individuals posing as traditional healers (for lucrative purposes) has increased with the popular demand of this practice. Often these untrained individuals harvest plant materials from areas where plants may have been exposed to pollutants. The latter is not uncommon and it has been found that plants harvested indiscriminately are often contaminated with toxic substances, microbiological contaminants and heavy metals [Chan (2003)]. This is an issue of great concern because of the difficulty experienced in the regulation of plant products being sold on the formal and informal markets and the consequential poisoning of unsuspecting patients.

Another method of malpractice is the adulteration of medicinal plants — the deliberate addition of biologically active compounds. It has been documented that several plant products have been "enhanced" by the addition of western pharmaceuticals with severe toxicological consequences [Snyman *et al* (2005); Yee *et al* (2005)]. The fact that traditional medicines are complex mixtures of compounds should be considered since it increases the possibility for interaction with westernized medicines [Ernst (2000)]. Several herbal remedies are renowned for interacting with pharmaceuticals, for example, garlic and ginseng interact with wafarin, anti-platelet drugs and non-steroidal anti-inflammatory drugs. St. John's wort inhibits the effects of digitalis and Feverfew inhibits the effects of anti-serotonin dugs [Valli and Giardina (2002)].

The westernization of the African continent has led to an increase in the occurrence of epidemiological diseases. The incidence of "western" diseases, particularly AIDS, cancer and diabetes, has increased dramatically over the last 20 years and accordingly traditional medicines have been used to treat these diseases. In traditional medicine, *S. frutescens* and *Lobostemon trigonus* are used to treat wasting in HIV patients [Harnett *et al* (2005)], *Bridelia micrantha* is used to treat diabetes [Iwalewa *et al* (2007)], and *S. frutescens* is used to treat cancers [Chinkwo (2005)]. On the other hand, traditional medicines have gained popularity in first world countries. Many consumers prefer natural remedies to western pharmaceuticals in the treatment of disease such as the use of *S. frutescens* for the treatment of cancers and diabetes. In the United States of America the estimated market value for plant based medicines is approximately $36 billion and $70.5 billion in the Asian countries [Gerry (1995)]. The medicinal plant trade in South Africa alone generates approximately R465 million annually [Mander (1998)].

The demand for natural products for the treatment of diseases such as diabetes, ailments associated with the central nervous system (CNS) and cardiovascular disease has increased [Barnes (2008)]. South Africa is considered to be well positioned to investigate alternative medicines, due to its rich floral diversity and infrastructure. Traditional healers are also focusing their attention on modern diseases and the exploration of traditional medicines by various research groups indicates the beginning of intricate networks between traditional healers, research institutions and conservation organizations [Makunga *et al* (2008)].

2.2 Ethnopharmacological research

Chinese and Indian traditional medicines are examples of traditional health care systems that have been extensively documented over many centuries [Cragg & Newman (2001)]. Unfortunately this is not the case for African traditional medicines. The earliest publication regarding medicinal plants in Southern Africa is that by Watt and Breyer-Brandewijk which was first published in 1962. This publication was an extensive and comprehensive manuscript that documented the medicinal plants of the Eastern and Southern African countries and has been a noteworthy work of reference concerning traditional medicines [Watt & Breyer-Brandwijk (1962)].

African traditional knowledge has been passed from generation to generation in stories and folklore. Thus formal documentation is limited and this may have led to indigenous knowledge being lost. Factors which contribute to the fragility of the indigenous knowledge in South Africa include the strong influences from other cultures from different continents and urbanization [Van Wyk (2002)]. In an attempt to preserve this knowledge, more efforts are being made to document the traditional practices of various cultures in South Africa. However, it seems that limited information regarding these intricate health care systems have been brought to light. Recent publications on cultural groups in South Africa [Van Wyk & Gericke (2000); Van Wyk (2002)] have encouraged new interests in this field. Changes in the socio-political environment have sparked both interest and funding associated with investigating traditional medicines. The restructuring of the National Research Foundation (NRF) led to a marked increase in research in the fields of ethnopharmacology and ethnobotany which is reflected in the number of publications concerning traditional medicines in South Africa, as opposed to the rest of the African continent. This increase in publications in the Journal of Ethnopharmacology alone, over the last 30 years is shown in Figure 2.1.

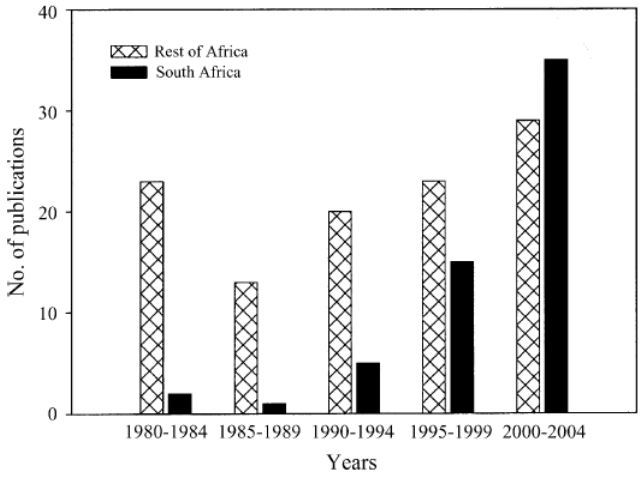


Figure 2.1: Contributions from South Africa and the other African countries in regard to traditional medicine research published in the Journal of Ethnopharmacology [Light *et al* (2005)].

The investigation of the medicinal potential of plants plays a major role in ensuring a safe product. Although most medicinal plants are assumed to be safe, this is not always the case and recent research has shown that plants used commonly in traditional medicines

and foods may display toxic, carcinogenic and mutagenic effects [Higashimoto *et al* (1993); Kassie *et al* (1996); De Sã Ferrira & Ferrão Vargas (1999)].

2.3 Safety and efficacy of traditional medicines

Plants that are frequently used in traditional medicines are considered to be safe for use due to assumptions routed in the history of treatments rather than medical fact. Research has identified numerous toxic plants used in traditional medicines, previously thought to be safe [Schimmer *et al* (1988)]. In South Africa, approximately 50 plants, commonly used by traditional healers, were tested for potential hazardous effects related to long-term usage. Both polar and non-polar plant extracts were tested for potential genotoxic effects by using assays such as the micronucleus test, the Ames test, the comet assay and the VITOTOX test [Taylor *et al* (2003)]. These studies showed that most plant extracts tested led to DNA damage, chromosome lagging in white blood cells or chromosome aberrations. There were also plants that caused frame shift mutations when assayed in *Salmonella* TA98 microsomal preparations. Interestingly the induction of genotoxicity was influenced by the extraction method, which varied between different traditional medicinal plants and the plant species [Elgorashi *et al* (2002); Elgorashi *et al* (2003)]. The micronucleus and Ames tests identified plants that exhibited anti-genotoxic effects by, for example, lowering the effects of mutagens significantly [Verschaeve *et al* (2004)]. These findings highlight the fact that the unregulated practice and distribution of medicinal plants could lead to acute poisoning.

It is commonly believed that few plants used in traditional medicines in South Africa cause serious toxicity [Arnold *et al* (2000)]. Poisoning is usually a direct result of misidentification, improper preparation, inadequate administration and incorrect dosage [Stewart & Steenkamp (2000)], often as a result of self medication [Popat *et al* (2001)].

Acute poisoning due to traditional medicines results in a mortality rate of between 8000 and 20 000 deaths per year with the variation between the two figures given being due to inadequate data collection [Thomson (2000)]. It is estimated that 43 % of the fatalities resulting from poisoning which were reported between 1991-1995 were due to traditional plant medicines, with the majority of poisoning cases remaining undocumented [Stewart *et al* (1999)]. This implies that the fatalities attributed to the use of traditional medicine,

is currently much higher than the estimated figure [Thomson (2000)]. In a recent case of organo-phosphate poisoning in a neonate caused by traditional medicines, a sample of the medicine administered to the neonate was confiscated. The sample was, however, never analyzed due to it being misplaced and the traditional healer was never prosecuted [Van Wyk & Els (2008)].

In many cases of acute poisoning, Western healthcare is often consulted too late as primary healthcare is often inaccessible due to the patient's location. In addition, hospitalization is often delayed since hospitals are often deemed to be culturally unacceptable. Traditional medicines are considered to be secret in some cultures and when regarded to be primitive by attending medical staff, patients shy away from hospitalization [Popat *et al* (2001)].

Although the inappropriate use of medicines in all healthcare systems is associated with significant health risks, these risks are of greater concern in the South African traditional healthcare system. A contributing factor is the manner in which plant medicines are sold. Plant materials, which include roots, leaves, bulbs and/or bark, are dried and semi-processed before they are sold. Taxonomically reliable characteristics become difficult to distinguish after plant materials have been dried leading to the misidentification of the plants which could ultimately lead to poisoning [Botha *et al* (2001); Cunningham (2001); Williams *et al* (2001)].

Other factors that could lead to safety issues regarding the prescribed plant material are cultivation techniques, exposure to pollutants and storage of the raw plant materials after harvesting [Fennell *et al* (2004a)]. Many medicinal plant harvest sites have been polluted by sewage and polluted water [Verster *et al* (1992)]. Consequently, the quality of the medicinal plants harvested from these sites is affected and the plant material contaminated with toxic substances, as mentioned earlier. The pollutants from the soil and the water are not the only pollutants that the plant materials are exposed to. These materials are often displayed openly in direct sunlight on the informal markets, shown in Figure 2.2, causing the material to be exposed to pollutants in the air, insects and microbes [Stafford *et al* (2005)]. In addition, traders and growers often overlook appropriate drying conditions which could lead to mould growth on the plant material,

causing the plant product to deteriorate [Whitten (1997); Ramakrishnappa (2002)]. In addition to plant deterioration, moulds and yeasts may cause opportunistic infections and are therefore especially dangerous in subjects with impaired immune systems. A recent investigation into the fungal and bacterial content of medicinal plants prescribed by traditional healers for HIV/AIDS patients showed high fungal and bacterial counts [Govender *et al* (2006)]. These counts exceeded the accepted microbial count in modern foods and supplements [Tadmor *et al* (2002)].

Figure 2.2: Informal market in Pietermaritzburg in South Africa [Street *et al* (2008)].

Although it is assumed that the conditions and duration of storage can influence the quality of the plant materials, few studies have addressed this issue. The effects of storage were assessed by investigating the biological activity of nine medicinal plants in terms of anti-bacterial and anti-inflammatory activities. Although the anti-bacterial activity remained unchanged, the anti-inflammatory activity diminished over time [Fennell *et al* (2004a)]. Studies investigating changes in the chemical composition of plant materials using thin layer chromatography [Stafford *et al* (2005)] concluded that phytochemical

stability varies between plants species. No recommendations regarding shelf life could be proposed based on these studies.

The limited availability of medicinal plant resources impacts the South African traditional healthcare system. Although the unregulated informal markets make plant materials accessible for many consumers, the plant materials sold in these markets are often of inferior quality, as previously mentioned. In addition, the vendors often harvest the plants from geographically different locations in the wild which leads to a variation of the compounds in the plant material [WHO (2003); Bopana & Saxena (2007)]. There have also been reports of alternative plants being sold instead of the scarce, preferred plant in spite of the negative effects this may have on the health of the consumer. It is therefore common to find cheap, easily accessible, alternative plant materials when a specific plant product is unavailable [Anyinam (1995)]. In an attempt to ensure a safe product for consumers, researchers have identified several compounds within traditional medicinal plant extracts.

2.4 Bioactive compounds in medicinal plants

A vast number of diverse compounds are found in plant materials with different structures, modes of action and functions. These compounds, also called secondary metabolites, are produced by the plant in addition to the primary metabolites which are essential for its survival. The secondary metabolites are produced to give these plants an evolutionary advantage [Van Wyk & Wink (2004)].

Although carbohydrates are common primary metabolites in plants, many secondary metabolites are produced through glycosylation of common carbohydrates. Pentose- and hexose sugars are combined to produce complex polysaccharides in plants —pectin, cellulose, hemilose and starch. Mucilage is a particularly important storage product produced by many plants and has been used in traditional medicine to aid digestion. It has also been used to soothe inflamed tissues by forming a protective membrane over the inflamed area [Van Wyk & Wink (2004)].

In the interest of this specific study, only compounds common to the Fabaceae family, of which *S. frutescens* is a member, will be considered and discussed.

2.4.1 Alkaloids

Alkaloids are plant products that include all nitrogen containing compounds that are not considered to be peptides, non-protein amino acids, cyanoglycosides, cofactors, primary metabolites, antibiotics, amines, glucosinolates and phytohormones. These compounds are found in animals, fungi, bacteria and in approximately 15 % of all plants. The pharmacological activity and/or toxicity of different alkaloids in humans and animals have been investigated, leading to the accumulation of information regarding their modes of action and their various target sites [Van Wyk & Wink (2004)].

Alkaloids are stored in tissues that play a role in the plant's reproduction and survival and are involved in the growing of bark, roots, young tissues, seeds and seedlings, flowers and photosynthetic plant tissue. It is common to find alkaloids in epidermal and sub-epidermal tissue in herbaceous plants where these compounds function as the plant's first line of defense against smaller threats, including insects. The alkaloid concentrations may vary in plants according to the sites where the plants are harvested. Furthermore, alkaloid synthesis is affected by a number of factors including secondary substitutions that may take place, whether transport by phloem is selective, the developmental stage of the plant, the stage of its annual cycle and nitrogen availability. These factors would contribute towards the medicinal potential of a plant and if a maximal alkaloid concentration is required, the harvesting time is an important consideration [Van Wyk & Wink (2004)].

Alkaloids often share the same amino acid precursor as neurotransmitters such as dopamine, serotonin, glutamic acid, gamma aminobutyric acid (GABA), histamine and adrenalin. Consequently, their structures are very similar to that of the neurotransmitters which enables them to compete for the same receptors as the neurotransmitters. Lipophilic and planar alkaloids act by intercalating DNA, while pyrrolizidine alkaloids have the ability to modify metabolism in the liver. Other alkaloids posses the ability to interfere with microtubule assembly and ultimately results in apoptosis [Van Wyk & Wink (2004)].

Although alkaloids may elicit effects by modulating enzymes involved in signaling pathways such as phospholipase C, adenylyl cyclase, protein kinases and phosphodiesterase, their main action is via their ability to target neurotransmitters such as

serotonin, GABA, peptides, glutamate and endorphins. Alkaloids may also inhibit enzymes such as monoamine oxidase and acetyl-choline esterase which are responsible for the deactivation of neurotransmitters after binding to their receptors. In addition, alkaloids may interfere with the uptake and release of neurotransmitters as well as that of K^+, Na^+ and Ca^{2+}-ATPases in synaptic and pre-synaptic vesicles [Van Wyk & Wink (2004)].

2.4.2 Non-protein amino acids

Proteins are made up of different combinations of twenty essential amino acids. Although these amino acids are the only building blocks for proteins, approximately 600 non-essential amino acids have been discovered in plants. The non-essential amino acids do not form part of proteins and are therefore referred to as non-protein amino acids (NPAAs). Monocotyledonous and Fabaceae families are known to contain high levels of NPAAs in organelles such as rhizomes and seeds. Since the structures of NPAAs are similar to that of essential amino acids these compounds may interfere with metabolic processes by acting as anti-metabolites or anti-nutrients. NPAAs have the potential to interfere with protein biosynthesis by firstly, inhibiting amino acid uptake and secondly, through disrupting the translation process resulting in faulty proteins [Van Wyk & Wink (2004)].

Canavanine (Figure 2.3) is a NPAA that is commonly found in legumes. It is an arginine antagonist and has well-documented anti-cancer properties [Van Wyk & Wink (2004)].

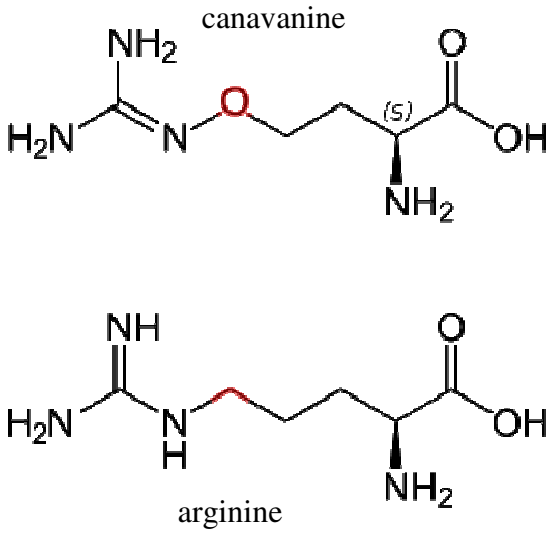


Figure 2.3: Structure of canavanine and arginine.

2.4.3 Cyanogenic glycosides

Members of the Rosaceae, Araceae, Fabaceae and Gramineae families are known to contain cyanogenic glycosides. The cyanogenic glycosides play an important role in the defence mechanism of plants when damaged by a herbivore. If cellular compartmentalization damage has occurred, cyanogenic glycosides from the vacuole bind to active β-glucosidases. The enzyme has a broad specificity and hydrolyses the cyanogenic glycosides to produce 2-hydroxynitrile, which is cleaved by hydroxynitrile lyase resulting in a ketone or an aldehyde and hydroprussic acid (HCN). HCN is a highly toxic compound that has been responsible for many deaths in humans and animals. The toxic effect is attributed to HCN's ability to inhibit ATP production thus inhibiting enzymes in the respiratory chain. Enzymes containing heavy metals ions are also a target for inhibition by HCN [Van Wyk & Wink (2004)]. Figure 2.4 shows the reactions leading to the release of HCN by cyanogenic plants [Francisco & Pinotti (2000)].

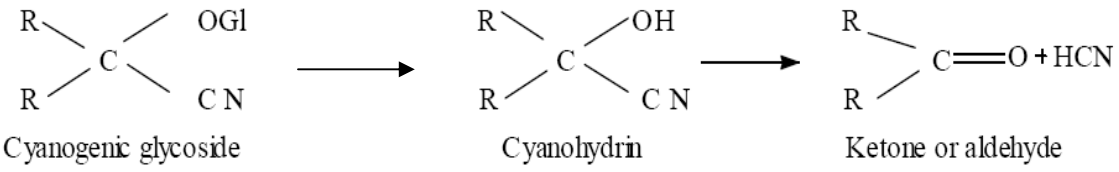


Figure 2.4: Reactions leading to the release of HCN [Francisco & Pinotti (2000)].

2.4.4 Terpenoids

Terpenoids have been identified in many plant products. Although their main components appear to be simple, these molecules are complex due to secondary ring structure formations and the addition of chemical groups. Terpenoids are classified according to the number of carbon atoms in their structure.

Firstly, monoterpenes (10 carbon atoms) and sesquiterpenes (15 carbon atoms) are lipophilic compounds that are stored in organelles such as trichomes, oil cells, resin channels or even other deceased cells. Due to their volatile nature, terpenoids may be isolated through the process of steam distillation. Diterpenes (20 carbon atoms) have complex ring structures and several of these compounds are renowned for their toxicity and ability to cause cancers by mimicking signalling molecules [Van Wyk & Wink (2004)].

Plant steroids and triterpenes are compounds derived from 30-carbon compounds and although they have been known to appear as free compounds, glycosylation is common as is the case with saponins. The major difference between the abovementioned compounds and saponins is that plant steroids and triterpenes are highly lipophilic while saponins are hydrophilic. Saponins are stored in the vacuole in their inactive forms which are triterpene bidemosides or furanostanol glycosides and are converted to active triterpene monodesmosides or spirostanol glycosides when the plant is attacked by another organism. These active forms are known to display membrane activity. Both steroidal and triterpenoid saponins have been isolated from *Asparagus africanus, Celmisia spectablilis* and *Capsicum frutescens* [Sparg *et al* (2004)]. Steroidal and triterpenoid saponin structures are shown in Figure 2.5.

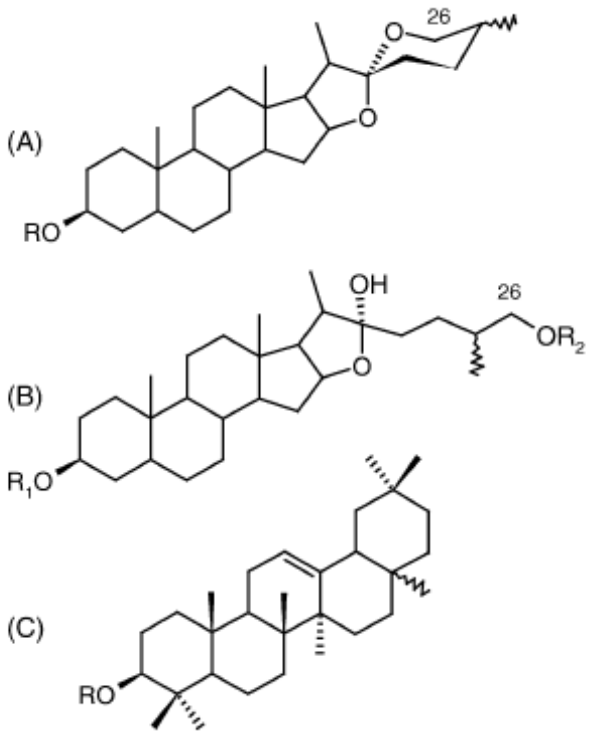


Figure 2.5: Skeleton structures of (A) steroidal spirostane, (B) steroidal furostane, and (C) triterpenoid saponins. Sugar moieties are represented by R [Sparg *et al* (2004)].

Cardiac glycosides also fall under the class of steroidal saponins. These medically useful compounds are known to inhibit K^+, Na^+-ATPase and are used to treat heart conditions even though they are considered to be strong toxins. The cardiac glycosides vary in structure which, while not affecting their binding or their rapid action, does affect their uptake. The hydrophilic molecules are taken up slowly with the more lipophilic molecules diffusing into the body readily. In addition, the lipophilic molecules are retained in the body for longer periods because of their ability to bind to plasma proteins. These molecules are stored in adipose tissue [Van Wyk & Wink (2004)].

Tetraterpenes (40 carbon atoms), which include the carotenoids, are lipophilic molecules that are always associated with biological membranes (3 common carotenoids shown in Figure 2.6). They have many functions in plants which include serving as additional pigments in chloroplasts and providing the colour of many fruits and flowers, attracting pollinators. In animals, carotenoids are the precursors for vitamin A.

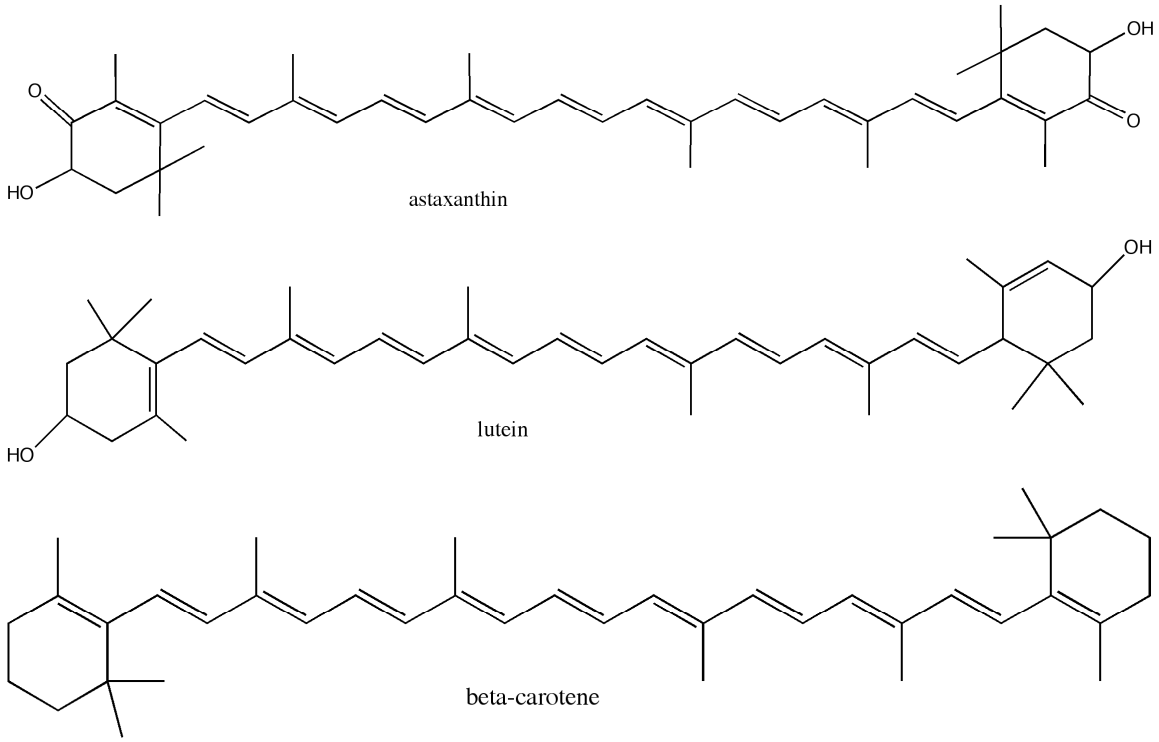


Figure 2.6: Structures of common caretanoids.

All the above-mentioned terpenoids have similar biological activities with biological membranes being their main target. By increasing the efflux of ions, receptors and metabolites, they increase membrane fluidity causing cell leakage and ultimately cell death. Signal transduction is also disturbed by the increased efflux of ions. Due to the ability of the tetraterpenes to cross the blood-brain barrier, these compounds are often used as sedatives and analeptic drugs. Since tetraterpenes also elicit anti-bacterial actions, extracts of essential oils are used to treat infections and disorders of the respiratory tract [Van Wyk & Wink (2004)].

2.4.5 Phenolic compounds

A number of compounds found in natural plant products are categorized as phenolic compounds. These compounds include phenylpropanoids, tannins, flavonoids and courmarins of which the precursor compound is phenylpropanoid (a phenylalanine and tyrosine derivative). The precursor can also occur as simple compounds differing only to the extent of methoxylation and hydroxylation [Van Wyk & Wink (2004)].

The conjugation of two phenylpropanoid molecules yields tannins which are hydrophilic and are known to cause a bitter taste in beverages, especially wines and teas. Tannins are primarily used in the process of leather tanning due to their acidic nature.

Flavonoids are produced when a polyketide condenses with a phenylpropanoid. These compounds are stored in the vacuole and are primarily secondary metabolites of fruits and flowers. Colour pigments in fruits and flowers can be attributed to the flavonoids which can vary from pink to dark blue. Flavonoids have two characteristic aromatic rings that often have methoxyl- and phenolic hydoxyl groups attached to them (Skeleton structures and classification hierarchy are shown in Figure 2.7). These groups have the potential to influence various molecular targets by interacting with proteins through ionic bonds. Many of the biological activities of phytomedicines have been attributed to flavonoids, although there is little data supporting these assumptions [Van Wyk & Wink (2004)].

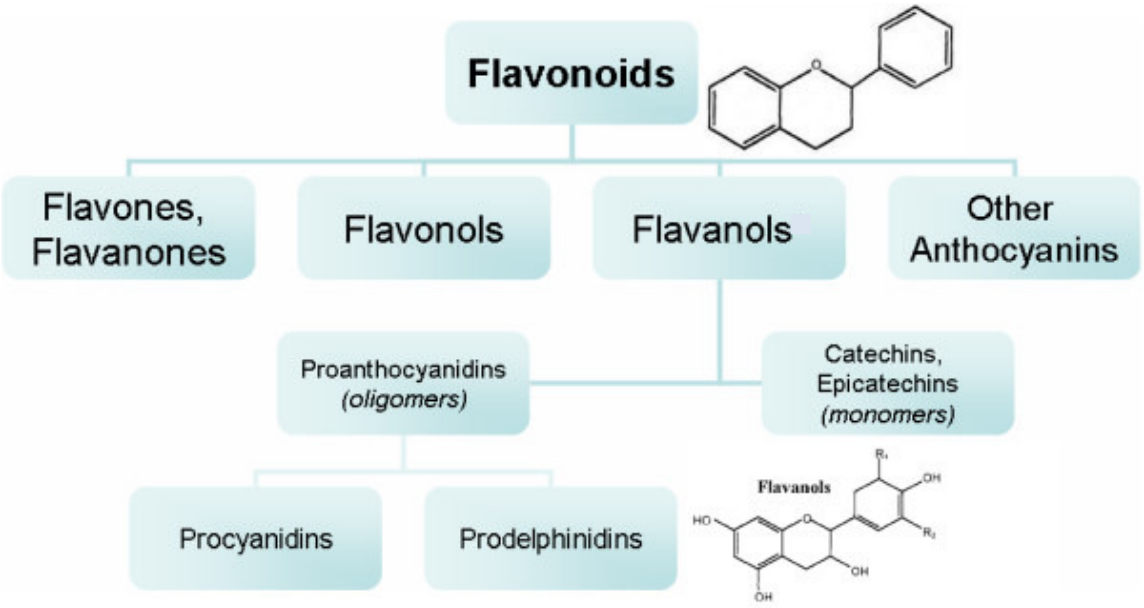


Figure 2.7: Structure backbones and classification hierarchy of flavonoids [reproduced from Ding *et al* (2007)].

Coumarins and furocoumarins are stored in the vacuole and released upon tissue damage as active coumaringlycosides. After hydrolyses by β-glucosidase, isomeration occurs, yielding simple coumarins. When furocoumarins are exposed to UV light, these compounds have the potential to form cross-links between DNA bases and proteins.

Therefore these compounds play a major role in a plant's defence mechanism and are commonly used to treat vitiligo and psoriasis in humans [Van Wyk & Wink (2004)].

2.4.6 Polyketides

Polyketides are acetate derivatives that may be present in plant products either as secondary metabolites, like anthraquinones, or bound to flavonoids. Anthracene derived secondary metabolites are usually represented as mono- and diglycosides in plants. These compounds disturb intestinal adenylyl cyclase and K^+, Na^+ ATPases which stimulates gut motility, increasing the water content of faeces ultimately resulting in a strong laxative effect. Phenolic hydroxyl groups present in anthraquinones have the ability to interact with DNA and various proteins in the human body possibly stimulating mutagenesis [Van Wyk & Wink (2004)].

2.5 Conclusion

South Africa boasts a rich floral diversity with a long history of plants used in traditional medicines. These medicines have served as a primary healthcare system for a large percentage of the population and continue to do so despite westernization.

Traditional healers are important figures in many cultures and are responsible for the competent recommendation and distribution of traditional medicines. Several laws have been implemented to secure the quality of healthcare provided by these healers. However, since traditional medicines have not been standardized, regulation is problematic. A major concern regarding traditional healers is that uninformed individuals pose as traditional healers for lucrative purposes often leading to the poisoning of patients.

The increase in the popularity of traditional medicines has led to an increase in cultivation and commercialization. Validation of the use of certain plants for medicinal purposes and the assessment of the safety and efficacy of medicinal plants is therefore critical. These factors have raised scientific interest leading to the accumulation of mostly preclinical data.

Most South African studies investigating medicinal plants are limited to a few biological activity studies and short clinical trials. These studies are insufficient and have not contributed much to the assessment of safety and efficacy of medicines people use daily.

South African communities that rely on traditional medicines can benefit greatly from support by the government in this regard. The fact that the majority of the South African population still relies on traditional medicines, with or without scientific guidance, screening for stable compounds or the assessment of efficacy and safety, is a health concern. The integration of programs that offer these individuals an understanding of the possible effects of medicinal plants, especially those obtained from the informal markets, could assist the prevention of poisoning. Ultimately, more research into the bioactivities of traditional medicinal plants is required.

Considering the economic impact that the medicinal plant trade has, not only in South Africa, but also in the USA, Europe and Asia, the medicinal plant trade should not be taken lightly. Even though the medicinal plant trade is primarily informal in South Africa, greater commercialization holds great economic potential and continued support of the medicinal plant trade will contribute towards the primary healthcare provided to many consumers.

Several Southern African traditional medicinal plants, including *S. frutescens*, have recently been the emphasis of scientific investigations. Anecdotal claims of the potential of *S. frutescens* to cure AIDS and cancer have raised interest in this plant and its traditional medicinal uses. The central role of *S. frutescens* in Southern African traditional medicine and the supporting scientific data are discussed in the following chapter.

Chapter 3

Sutherlandia frutescens

S. frutescens is a Southern African shrub which has been used extensively in traditional medicine. Decoctions made from this plant have been used by the indigenous people to alleviate the symptoms of many diseases such as cancer, diabetes, stress and anxiety, wounds, pain, fever, infections, tuberculosis, peptic ulcers and wasting in HIV and cancer patients.

Today many indigenous people still rely on traditional medicines for their primary healthcare even after the Westernization of many African countries. Within the traditional medicinal system *S. frutescens* remains a popular medicinal plant, which has led to increased cultivation and commercialization. Consequently there has been an increase in research interest and development of *S. frutescens* products.

3.1 Taxonomy, description and distribution

S. frutescens R.Br (Fabaceae) is commonly known as cancer bush. The *Sutherlandia* genus is named after James Sutherland who was the first superintendant of the Edinburgh Botanic Garden. The specie name, *frutescens,* means bushy in Latin. Besides cancer bush, *S. frutescens* is also known as umnwele, balloon pea, Insiswa, Phetola and Mukakana, turkey flower, gansies and belbos.

The Fabaceae family is a group of pod-bearing, flowering plants that are found all over the world. This family consists of 12 000 morphologically different species in 600 genera. There are about 1 300 species (134 genera) of this family found in Southern Africa. Original field studies suggested that there were six species of *Sutherlandia*, but more recent evidence shows that only two significantly different species exists — *S. frutescens* and *S. tomentosa* [Moshe (1998)]. Goldblatt and Manning (2000) suggested *Sutherlandia* to be part of the genus *Lessertia* DC, as these species are similar and most probably closely related. However, no genetic or morphological analysis has been documented to validate such a claim. Subsequently, the use of the names *Lessertia frutescens* and *S. frutescens* are both correct [Goldblatt & Manning (2000)]. The notion that some hairy subspecies of *S. frutescens* are the result of crossbreeding between *S.*

frutescens and *S. tomentosa* was supported by an electrophoretic study done by Moshe *et al* (1998). Monomorphic and polymorphic enzymes (listed in Table 3.1) were used to determine genetic variation between *S. frutescens* species. The data did not suggest any significant genetic diversity since allozyme patterns could not account for the morphological diversity within the species.

Table 3.1: Enzymes used to determine genetic variation between species of *S. frutescens* are listed below [reproduced from Moshe (1998)].

Enzyme	**Enzyme locus**
Adenylate kinase	AK
Esterase	EST-1, EST-2, EST-3
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH-1, GAPDH-2
Glucose-6-phosphate isomerase	GPI-1, GPI-2
Isocitrate dehydrogenase	IDH
Leucine aminopeptidase	LAP
Malate dehydrogenase	MDH-1, MDH-2, MDH-3
Menadione reductase	MNR-1, MNR-2, MNR-3
Manose-6-phosphate dehydrogenase	MPI
Peptidase, substrate:	
Leucine-analyl	PEP-C-1, PEP-C-2
Leucylglycylglycine	PEP-B-1, PEP-B-2, PEP-B-3
Leucyl-tyrosine	PEP-S-1, PEP-S-2
Peroxidase	PER-1, PER-2
6-Phosphogluconate dehydrogenase	PGDH-1, PGDH-2
Phosphoglucomutase	PGM-1, PGM-2
Superoxide dismutase	SOD-1, SOD-2

The lack of accordance between the morphological diversity and the enzyme patterns are said to be attributed to the breeding mechanism of these plants. Geographical isolation is also thought to play a major role in the morphological differences in these species. The geographical distribution of the *Sutherlandia* taxa is shown in Figure 3.1. The *Sutherlandia* taxa are said to be pollinated in two ways: self pollination and pollination by birds, specifically the malachite sunbird (*Nectarinia famosa*). It has been suggested that flowers may self pollinate in situations when the population is small and isolated. This results in population growth which subsequently may attract pollinators. Pollinators carry genetic material to other populations that are geographically isolated and thus allele recombination between taxa is increased. This theory, however, does not agree with the idea that there could be six different *Sutherlandia* taxa. The lack of distinctive alleles between these taxa make it impossible to discriminate between them and therefore the only characteristics presently used to discriminate between taxa are the shape of the pods, habitat, pubescence and shape of the leaves, as well as the orientation of the fruit. Due to a lack of genetic variation, the Southern African genus is suggested to be part of only one taxon, *S. frutescens* [Moshe (1998)].

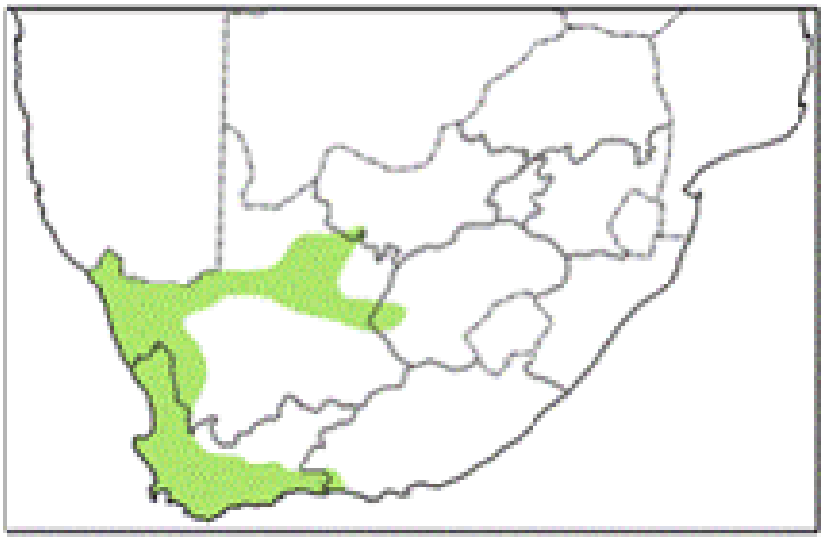


Figure 3.1: Distribution of *Sutherlandia frutescens* in Southern Africa

S. frutescens is a perennial, erect shrub with a height varying from 0.2 m-2.5 m. The *S. frutescens* scrubs have compound leaves, bladder-like pods and bright red flowers. As previously mentioned, the flowers attract birds to aid pollination [Van Wyk & Wink

(2004)]. The plants have been cultivated for use as garden ornamentals for a number of years and have recently been cultivated for commercial purposes (Figure 3.2).

Figure 3.2: Characteristic leaves, flowers and pods of *S. frutescens*.

These plants are easily grown from the seeds collected from the pods. In the wild, the plants seed profusely and the seedlings sprout rapidly around the parent plant. In the case of *S. frutescens* grown commercially, the seeds are planted in early spring and will flower the following year. *S. frutescens* tolerates most soil types and is resistant to frost. The plants flourish in full sun and well drained soils, and perform at their best when they receive ample moisture in winter and spring time [Diederichs (2006)].

S. frutescens has many uses in traditional medicines. It has been used as a herbal remedy for the relief of a wide variety of ailments with little or no scientific appraisal [Smith (1895); Watt & Breyer-Brandewijk (1962)]. In traditional medicine, decoctions made from this shrub are usually made by boiling the dried plant material (mostly leaves and stems) in water, straining the mixture and drinking the infusion. Although this is the most

common method of preparation and form of ingestion of *S. frutescens* plant material, it is also commercially available in tablet form.

3.2 Safety

S. frutescens is still commonly used by a large percentage of the South African population in spite of the lack of information regarding the safety and efficacy of the plant. Although *S. frutescens* has a long history of safe use as a traditional medicine there are many questions surrounding this plant and its medicinal use that remain unanswered.

Known side effects that have been associated with the medicinal use of the plant are mild diarrhea, dryness of the mouth, mild diuresis and occasional dizziness [Mills *et al* (2005)]. In terms of safety, Ojewole *et al* showed that crude extracts of *S. frutescens* are relatively safe for use by mammals and determined the lethal dosage of crude aqueous extracts in mice as 1280 ± 71 mg of extract per kilogram body weight [Ojewole (2004)]. The highest recorded dosage of *S. frutescens* ever taken by humans was 5 g of leaf material twice daily, showing no negative effects over a period of 6 years [Van Wyk & Albrecht (2008)]. A study of the safety of *S. frutescens* in Vervet monkeys concluded that an amount of 300 mg twice daily is safe to use and is now the recommended dosage of commercially available products [Seier *et* al (2002)]. The effects of *S. frutescens* on pregnant or lactating woman have not yet been determined. A clinical trial consisting of 12 treatment subjects and 13 control subjects, found no significant effects when physical, vital, biomarker and blood indices were assessed. The six physiological variables that were found to be significantly different were respiration rate, platelet count, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and total protein and albumin levels. The treatment group showed significantly higher MCH, MCHC, platelet counts, total protein and albumin levels compared to the control group. The control group exhibited a higher respiration rate than the treatment group. Improved appetite and weight gain was also observed in the treatment group. Interestingly, the *S. frutescens* biomarker canavanine was not detected in the plasma samples of the treatment group. It was concluded that 400 mg of dried leaf material of *S. frutescens* twice daily had no adverse effects. [Johnson *et al* (2007)].

3.3 Compounds found in *S. frutescens* extracts

The medicinal properties of plants are usually attributed to bioactive compounds within these plants called secondary metabolites. Although secondary metabolites do not play a role in the primary functions of the plant (photosynthesis, respiration, etc.) and are therefore not essential for the survival of the plant, these compounds give the plant an evolutionary advantage by attracting pollinators or deterring potential attackers. These compounds vary in structure and composition and are found within a variety of species. Examples of secondary metabolites found in plants with medicinal applications are ginsenosides found in Ginseng [Dewick *et al* (1997); Huang (1999)], hypericin in St. John's wort [Schultz *et al* (1998); Upton *et al* (1998)], ginkolides in *Ginkgo biloba* [Dewick *et al* (1997)] and kavapyrones found in *P. methyscum* [Schultz *et al* (1998)]. Although there are several examples of secondary metabolites that elicited medicinal effects, the bioactive compounds in the majority of traditional medicinal plants are unidentified.

Several bioactive compounds have been identified in *S. frutescens* which may contribute to the medicinal effects elicited by these plant extracts. *S. frutescens* leaves have been shown to have high levels of free and bound amino acids. Of the free amino acids identified, arginine (0.5-6.7 mg/g), proline (0.7-7.5 mg/g) and asparagine (1.6-3.0 mg/g) are present in the highest concentrations. These amino acids make the plant a note worthy and appetizing fodder plant [Van Breda & Barnard (1986); Shearing & Van Heerden (1994); Le Roux *et al* (1994)].

The seeds of the Fabaceae family are known to contain NPAAs of which L-canavanine is present in *S. frutescens* leaves in high concentrations (from 0.42 mg/g – 14.5 mg/g) [Moshe (1998)]. L-canavanine has been linked to the plant's anti-cancer properties. *S. frutescens* has a long history of use against cancers and appears to be the first reported medicinal plant to contain L-canavanine (Figure 3.3). This compound also exhibits anti-viral and anti-retro viral properties [Tai *et al* (2004); Van Wyk *et* al (1997)].

GABA (Figure 3.3) is another NPAA found in *S. frutescens* extracts. This compound is an inhibitory neurotransmitter to which the bioactivity of *S. frutescens* extracts in the treatment of psychiatric disorders, such as stress and anxiety could be attributed. In

addition to this activity, Ortega *et al* (2003) showed that GABA is also able to inhibit the migration of tumor cells.

In the 1960's a cyclitol was isolated from *S. frutescens* extracts by various groups and identified as pinitol (Figure 3.3) [Brümmerhoff (1969); Snyders (1965); Viljoen (1969)]. Various studies have been conducted to assess the bioactivity of the compound. Pinitol was found to induce an insulin-like effect in the body, causing blood sugar levels to drop thereby increasing the availability of glucose for cellular metabolism. The latter activity indicates the importance of pinitol in *S. frutescens* relating to its traditional uses for treating diabetes and inflammation [Bates *et al* (2000)]. Studies confirming the anti-diabetic effects of *S. frutescens* were conducted by Chadwick *et al* (2007) and MacKenzie *et al* (2009) and are discussed in section 3.4.3. Earlier studies, conducted by Ostlund and Sherman (1996), showed that pinitol has potential for the treatment of wasting in AIDS and cancer patients.

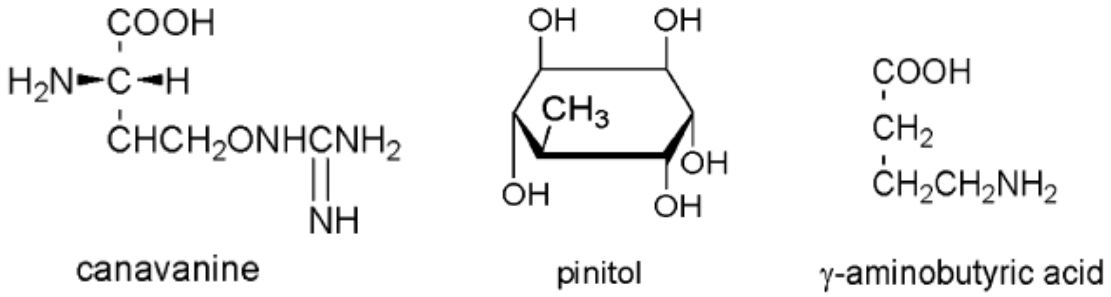


Figure 3.3: Compounds commonly found in *Sutherlandia frutescens* extracts.

Some of the more complex compounds present in *S. frutescens* include the triterpenoids and the flavonoids. Although triterpenoid saponins have been detected in *S. frutescens* extracts, the exact chemical structures remain unknown [Brümmerhoff (1969); Snyders (1965)].

Fu *et al* (2008) recently identified and isolated sutherlandiosides A-D (1-4) from *S. frutescens* extracts. The structures of these compounds were determined using x-ray chrystallography and are shown in Figure 3.4. Compound 1 is a secocycloartane with a 7, 10 oxygen bridge, whereas compounds 2, 3 and 4 represent cycloartanes with C-1 ketone

functionality [Fu *et al* (2008)]. As many as 56 other triterpene glycosides have been detected in *S. frutescens* extracts and it has been reported that the concentrations of these compounds vary according to geographic location of the plants [Van Wyk & Albrecht (2008)]. The chemical structures of these compounds have not yet been elucidated. Several of the biological activities elicited by *S. frutescens* extracts are claimed to be due to the presence of flavonoids. However, these flavonoids have not yet been identified and their structures are unknown [Moshe (1998)].

Several long chain fatty acids and polysaccharides are also present in *S. frutescens* leaf extracts. The exact compounds and their structures are yet to be determined [Tai *et al* (2004)].

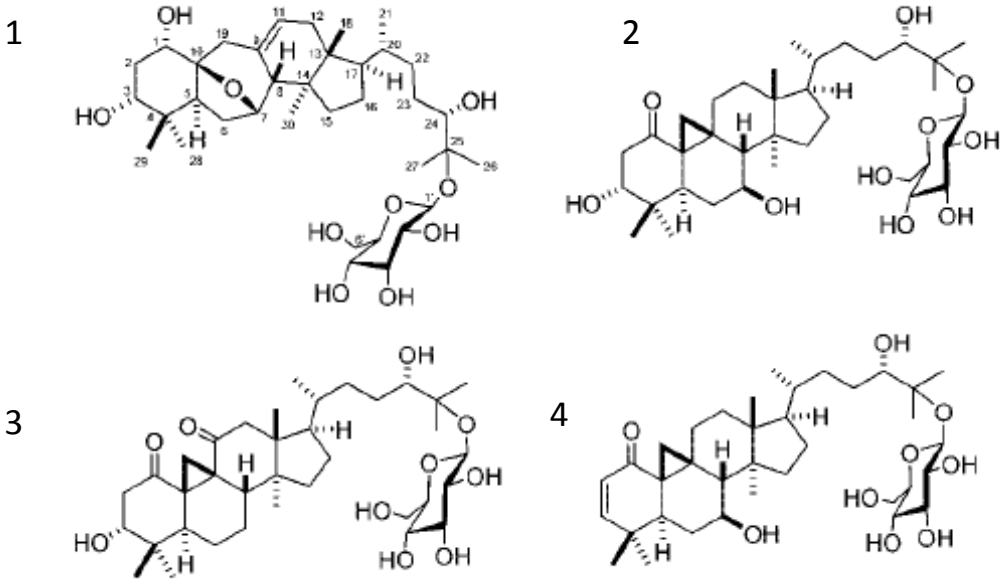


Figure 3.4: Sutherlandiosides 1-4 isolated from *S. frutescens* extracts [Fu *et al* (2008)].

3.4 Pharmacological effects of *S. frutescens*

The above-mentioned compounds found in *S. frutescens* are known to elicit specific pharmacological effects and recent scientific interest has led to the identification of several biological targets.

3.4.1 Cancer

S. frutescens was named cancer bush because the Khoi and the San used decoctions from this plant to treat internal cancers. Several other anecdotes describe improvements in the quality of life and survival rate of cancer patients who consumed *S. frutescens* infusions daily [Smith (1895); Van Wyk & Wink (2004)]. The use of *S. frutescens* as an anti-cancer agent could be attributed to the presence of canavanine. Although high concentrations of canavanine are known to be toxic and may possibly induce autoimmune diseases in prone individuals, no toxicity has been associated with canavanine at the concentrations found in *S. frutescens*. This could be attributed to either biotransformation or metabolism, or both, of canavanine by the cytochrome P450 system [Seier *et* al (2002)]. In addition triterpenoids present in *S. frutescens* exhibit structural similarity to other cycloartane-type triterpenoids that have documented chemopreventative activities [Kikuchi *et al* (2007)].

In 2004, Tai *et al* showed that ethanolic extracts elicited an anti-cancer effect by inhibiting the proliferation of several tumor cell lines. These researchers were the first to show that proliferation was inhibited in a dose dependant manner in the presence of *S. frutescens.* The ethanolic extract was diluted between 200 times and 1200 times and these dilutions inhibited the proliferation of MCF7, HL60, Jurkat and MDA-MB-468 cells (cancer cell lines) by up to 50 %. Although gas chromatography (GS) showed the presence of canavanine, arginine and GABA in these extracts, canavanine was thought to be responsible for the anti-proliferative action observed in the tumor cells. Canavanine could, however, not be responsible for the anti-proliferative effect since the addition of arginine, a canavanine antagonist, did not disturb the proliferative effect of the tumor cells.

Anti-inflammatory agents are known to exert chemopreventative activities through inhibiting cyclooxygenase (COX)-2, a rate limiting enzyme engaged in prostaglandin biosynthesis and the inflammatory response. It has been shown that the over-expression of COX-2 causes an increased susceptibility to tumor formation in genetically engineered mice, whereas COX-2 knockout mice show no promotion of tumor formation [Muller-Decker *et al* (1962); Tiano *et al* (2002)]. Studies have also shown that several COX-2 inhibitors have the ability to prevent cancer and thus support the theory that COX-2 plays

a role in tumor formation [Kakizoe (2003); Chun *et al* (2004)]. By applying methanolic extracts of *S. frutescens* to the skin of mice, 12-O-tetradecanoylphobol-13-acetate (TPA) induced expression of COX-2 was inhibited. The extracts reduced the TPA stimulated catalytic activity of extracellular signal regulated protein kinase (ERK). ERK regulates the activation of the transcription factors that mediate COX-2 induction in eukaryotes. The suppression of DNA binding to NF-κB inhibits TPA-induced COX-2 expression and this action is thought to contribute to the chemopreventative properties of *S. frutescens* [Na *et al* (2004)]. Kikuchi *et al* (2007) investigated the chemopreventative activity of 48 synthetic and natural cycloartane triterpenoids. The study showed that cycloartanes, with structures similar to that of the sutherlandiosides, were the most potent inhibitors of mouse skin carcinogenesis.

A study in 2005 showed that aqueous extracts of *S. frutescens* induced apoptosis in human cervical carcinoma cells and in Chinese hamster ovary cells [Chinkwo (2005)]. DNA fragmentation, chromatin condensation and phosphatidyl serine externalization are signs that indicate apoptosis and were observed in these cells after the addition of the extract. The apoptotic effects observed with the *S. frutescens* extracts were compared to effects observed with known apoptotic agents and confirmed with flow cytometric analysis [Steenkamp & Gouws (2006)].

Stander *et al* (2002) demonstrated the effect of ethanolic extracts of *S. frutescens* on gene expression, cell morphology and cell numbers in human breast adenocarcinoma cells. This study showed that exposure to 1.5 mg/ml extract over a 24 hour period decreased malignant cell numbers up to 50 %. Apoptotic signs such as cytoplasmic shrinking, apoptotic bodies and membrane blistering was observed.

Dichloromethane extracts revealed the anti-mutagenic properties of *S. frutescens* in an Ames test using *Salmonella typhimurium* TA98 and TA100 bacterial strains in the presence of S9, which is a metabolic activator. These researchers concluded that the dichloromethane extracts are potentially chemopreventative and anticarcinogenic [Reid *et al* (2006)]. Chen (2007) demonstrated that the growth of human prostate tumor cell lines was inhibited by polar and non-polar extracts in a dose dependant manner.

3.4.2 Pain and inflammation

Several anecdotes have indicated that *S. frutescens* extracts possess anti-inflammatory activity. Studies by both Kundu *et al* (2005) and Na *et al* (2004) showed that *S. frutescens* extracts inhibited the expression of COX-2. This is significant in regard to the inflammatory response since the expression of COX-2 is induced by cytokines at the site of inflammation and is responsible for the biosynthesis of prostaglandin. There are, however, two isoforms of the COX enzyme, COX-1 and 2. COX-1 is constitutively expressed in tissues such as the kidney and the stomach and is responsible for the biosynthesis of prostaglandins that are engaged in homeostasis, whereas the COX-2 enzyme is induced by inflammatory cytokines [Raz *et al* (1988)]. Since the 1970's nonsteroidal anti-inflammatory drugs (NSAIDs) have been used to treat inflammation through the inhibition of the biosynthesis of proinflammatory prostaglandins. The NSAIDs, however, are non-selective towards the two COX isoforms and inhibit the expression of both COX-1 and COX-2. This implies that the therapeutic use of NSAIDs for the treatment of inflammation arises from the ability of these drugs to inhibit the inducible COX-2 enzyme in inflamed tissues, whereas the toxic effects associated with the use of NSAIDs can be attributed to the inhibition of the constitutive COX-1 enzyme [Seibert *et al* (1994)]. Na *et al* (2004) and Kundu *et al* (2005) showed that topical application of *S. frutescens* extracts to mouse skin inhibits TPA induced COX-2 expression. As previously mentioned, TPA decreases the catalytic activity of ERK resulting in the inhibition of transcription factors that mediate COX-2 expression

In a study conducted by Ojewole in 2004, *S. frutescens* extracts displayed significant anti-inflammatory potential. In this study, fresh egg albumin induced paw edema was used as a model to assess the anti-inflammatory effects of the extracts. It was shown that the administration of *S. frutescens* root extracts significantly reduced the inflammation caused by the fresh egg albumin. Furthermore, the analgesic effects of *S. frutescens* root extracts were assessed using the hot plate and acetic acid models for pain in mice. Diclofenac (100 mg/kg) and chloropropamide (250 mg/kg) were used to compare the effects of 500-800 mg/kg *S. frutescens* root extract. The *S. frutescens* root extract

significantly reduced thermally and chemically induced nociceptive pain stimuli in the mice.

During the inflammatory response, the increase in inflammatory mediators causes an increase in oxygen uptake. The increased oxygen uptake and the metabolism of molecular oxygen cause the accumulation of reactive oxygen radicals (ROR), such as superoxide. In the presence of an antioxidant, the ROR is converted to a less harmful compound which can be further metabolized or eliminated from the body. If an imbalance between RORs and antioxidants occurs in favor of the former, oxidative stress may lead to cell damage or cell death [Sies (1985)]. Thus, inflammation may lead to tissue damage through oxidative stress.

Tai *et al* (2004) demonstrated that the *S. frutescens* extracts displayed no anti-oxidant potential. These researchers observed no significant suppression of liposaccharide induced NO production and no significant suppression of interleukin (IL)-1β and tumor necrosis factor (TNF)-α mRNA expression in mouse RAW 264.7 cells.

Fernandes *et al* (2004) suggested that there is a link between the antioxidant and anti-inflammatory activities. It was reported that hot water, acetone, polar and non-polar extracts of *S. frutescens* exhibited hydrogen peroxide and superoxide scavenging potential at concentrations as low as 10 µg/ml. The hydrogen scavenging potential of *S. frutescens* extracts was confirmed by Katerere & Eloff (2005) and Chen (2007).

3.4.3 Diabetes

S. frutescens is used to treat various metabolic disorders of which diabetes is the most common. In many cultures *S. frutescens* is the only medicinal plant used to treat this disease [Van Wyk *et al* (1997); Van Wyk & Gericke (2000)]. Although it has been suggested that the presence of pinitol in *S. frutescens* products is responsible for its anti-diabetic activity [Moshe *et al* (1998)], there is little pharmacological evidence to confirm this [Sia (2004)].

Bates and Bailey (2000) and Tai *et al* (2004) suggested that pinitol and L-canavanine are responsible for the anti-diabetic effects of *S. frutescens*. L-canavanine interferes with arginine uptake which is essential for NO production. NO is a reactive oxygen radical (ROR) that is produced by the effector $CD4^+$ and $CD8^+$ lymphocytes, which also produce

cytokines. Autoimmune diabetes often causes insulitis, which could result in the infiltration of dendritic cells and macrophages into the pancreatic islets leading to the stimulation of the lymphocytes [Jansen *et al* (1994); Kay *et al* (1997)]. Cytokines have the ability to destroy beta cells in two ways: (1) they could be toxic to the target cells [Rabinovitch & Suarez-Pinzon (1998)] or (2) produce RORs [Lotz *et al* (2000)]. The accumulation of RORs damages the beta cells since these cells have a very low capacity for scavenging free radicals. *S. frutescens* would diminish the effects of NO and thus prevent further damage to the beta cells. A study by Piganelli and co-workers showed that mice that were administered a ROR scavenger, such as a synthetic superoxide dismutase mimic, did not develop invasive insulitis when administered T-cell clones, which are known to cause diabetes. [Piganelli *et al (*2002)]. Also, high doses of canavanine are known to induce auto-immune diseases, such as systemic lupus erythamatosis, in prone individuals. No such effects have been associated with the canavanine found in *S. frutescens*.

Type 2 diabetes is characterized by malfunctioning pancreatic beta cells and insulin resistance. Beta cells will increase their insulin production to overcome insulin resistance, but in clinical conditions the insulin production is insufficient, resulting in glucose intolerance [Porte (1991)]. Consequently, the glycation reaction, also known as a glycosylation reaction, is initiated in neural and beta cells [Myint *et al* (1995); Tajiri *et al* (1997)]. Advanced glycation may stimulate the production of the oxidative stress marker, 8-hydroxy-2'-deoxyguanosine (8-OHdG) [Ihara *et al* (1999)]. Other studies suggested that the production of RORs in oxidative stress may induce apoptosis of the beta cells of the pancreas, thus inhibiting the transcription and translation of insulin [Matsuoka *et al* (1997); Kaneto *et al* (1996)].

S. frutescens has proven anti-oxidant potential [Fernandes *et al* (2004)] and could therefore be used to attenuate the damage to the beta cells. This action is suspected to be due to the pinitol and L-canavanine contents of *S. frutescens* extracts. A study by Bates *et al* (2002) showed that pinitol caused a reduction in blood glucose, an effect typically induced by insulin, when fed to hypo-insulinemic STZ-induced diabetic mice. In severe insulin resistant mice the same treatment did not result in an improved regulation of

insulin and blood glucose levels as was expected. A recent clinical trial concluded that subjects suffering from obesity and mild type 2 diabetes do not experience improved insulin sensitivity when receiving pinitol orally, although they did not appear to have any difficulty with the digestion of the compound [Bates *et al* (2000)].

There have been reports that *S. frutescens* has the potential to reduce plasma free fatty acids [MacKenzie *et al* (2009)]. *S. frutescens* was administered to rats on a high calorie intake diet to assess the effects of the extracts of *S. frutescens* on induced insulin resistance. After twelve weeks, the plasma free fatty acid concentration of the experimental rat group, fed *S. frutescens*, was lower than that of the negative control rats fed a normal diet. Not only were the free fatty acid levels lowered, the experimental rats did not develop insulin resistance and exhibited lower blood cholesterol levels overall. Another study, also using rats on a high fat diet, demonstrated the effects of *S. frutescens* on glucose uptake by administering [H^3] deoxy-glucose [Chadwick *et al* (2007)]. The rats that were fed *S. frutescens* exhibited a significantly increased glucose uptake in adipose and muscle tissue and decreased intestinal glucose uptake.

The above-mentioned effects suggest that *S. frutescens* has the potential to treat mild cases of type 2 diabetes, but further study is required to determine the modes of action and clinical efficacy.

3.4.4 HIV and AIDS

HIV/AIDS has become an enormous problem throughout the world, especially on the African continent. Many people have been infected by HIV and the mortality rate under South Africans is high [[Department of Health [South Africa] (2007)]. Global and national statistics are shown in Table 3. 2 A and 3.2 B, respectively.

Table 3.2 A: Estimated number of individuals infected with HIV/AIDS globally [reproduced from UNAIDS (2007)].

	Estimate	**Range**
People living with HIV/AIDS in 2007	33.0 million	30.3-36.1 million
Adults living with HIV/AIDS in 2007	30.8 million	28.2-34.0 million
Women living with HIV/AIDS in 2007	15.5 million	14.2-16.9 million
Children living with HIV/AIDS in 2007	2.0 million	1.9-2.3 million
People newly infected with HIV in 2007	2.7 million	2.2-3.2 million
Children newly infected with HIV in 2007	0.37 million	0.33-0.41 million
AIDS deaths in 2007	2.0 million	1.8-2.3 million
Child AIDS deaths in 2007	0.27 million	0.25-0.29 million

Table 3.2 B: Estimated number of deaths as a result of HIV/AIDS in South Africa [Department of Health [South Africa] (2007)].

Year of death	**Age (years)**					**Total**
	0-9	**10-24**	**25-49**	**50+**	**Unspecified**	
1997	35,441	22,639	92,829	160,076	5,574	316,559
1998	41,172	25,808	114,249	178,776	5,104	365,109
1999	41,835	27,690	129,916	178,892	2,704	381,037
2000	42,843	29,583	150,562	189,566	2,214	414,768
2001	44,902	31,452	173,226	202,009	1,920	453,509
2002	50,767	34,439	201,153	211,693	1,928	500,082
2003	56,708	37,499	229,418	227,778	2,796	554,199
2004	62,898	38,405	244,129	224,115	3,073	572,620
2005	67,715	38,389	250,897	233,101	3,235	593,337
2006	68,292	39,003	251,067	245,963	1,155	605,480
Increase 1997-2006	93 %	72 %	170 %	54 %	-79 %	91 %

Several claims have suggested that *S. frutescens* has improved the quality of life of AIDS and cancer patients by improving appetite and thus leading to increased body mass, lowering viral loads and increasing CD4 counts [Chaffy & Stokes (2002); Gericke *et al* (2001)]. Consequently, the National Institutes of Health and National Centre for Complementary and Alternative Medicine (NIH NCAM) funded a two phase clinical trial to asses the legitimacy of these claims. In 2007, the first phase of the study showed that *S. frutescens* had no adverse side-effects and was well tolerated in healthy adults [Johnson *et al* (2007)]. The second phase of the trial, investigating the effect in AIDS patients, is

currently underway. It has been shown that aqueous and organic *S. frutescens* extracts inhibit HIV target enzymes, such as HIV reverse transcriptase. The mechanisms for these inhibitory effects are still under investigation [Bessong *et al* (2006); Harnett *et al* (2005)]. The presence of canavanine is of interest since it has been documented to inhibit nitric oxide synthase. The latter suggests that canavanine could have potential to treat septic shock, which has often been associated with advanced AIDS [Anfossi *et al* (1999); Levy *et al* (1999)]. No clinical trials have been conducted regarding cross reactions between *S. frutescens* and anti-retroviral drugs.

3.4.5 Stress

Various vernacular names for *S. frutescens* implies its indigenous use in treating stress, grief and depression —musapelo (Basotho) means "to turn the heart around", insiswa (Zulu) means "to dispel darkness" and phetola (Tswana) means "it changes" [Van Wyk & Albrecht (2008)]. Psychological stress induces a cascade of reactions that leads to the activation of the HPA axis. Adrenocorticotrophic hormone (ACTH) is released from the pituitary gland and stimulates the steroidogenic cytochrome P450 enzymes in the adrenal cortex through a cascade of reactions to synthesize the glucocorticoids, cortisol and corticosterone. Cortisol elicits a negative feedback effect on ACTH secretion. This negative feedback inhibition ensures that the body returns to a homeostatic state after the stress stimulus has dissipated. Long-term exposure to glucocorticoids, often as a result of chronic stress, leads to the desensitization of the negative feedback mechanism, leading to many adverse effects (discussed in Chapter 4) [Reul *et al* (1998)]. An inflammatory stimulus could induce the HPA axis to produce ACTH and the physiological modifications that occur could prepare an organism for fighting an infection. Therefore, the corticosteroids initiate the production of cytokines and other inflammatory agents [Dantzer *et al* (1999)].

Many ailments related to stress are caused by the dysfunctioning of the endocrine system. The effects of *S. frutescens* on the steroid biosynthesis pathway have been investigated and it has been found that *S. frutescens* inhibits various cytochrome P450 enzymes which could ultimately reduce the production of cortisol. The effects of *S. frutescens* on immobilization stress in Wistar rats were investigated in four rat groups— two stressed

groups and two control groups. One of each of the stressed and the control groups received *S. frutescens.* Smith and Myburgh (2004) found that rats subjected to immobilization stress showed significantly increased corticosterone levels compared to the non-stressed rats. However, when the two stressed rat groups were compared, the group receiving *S. frutescens* showed significantly decreased plasma corticosterone levels. Interestingly, the two control groups both exhibited significantly increased testosterone levels compared to the stressed groups and the control group receiving *S. frutescens* exhibited significantly higher plasma corticosterone levels. Thus, the stressed rats receiving *S. frutescens* exhibited decreased plasma corticosterone levels compared to the stressed rats not receiving *S. frutescens*, and the control group receiving *S. frutescens* exhibited increased plasma corticosterone levels compared to the control group not receiving *S. frutescens* [Smith & Myburgh (2004)]. Prevoo *et al* (2004) showed that the inhibitory effect of *S. frutescens* on the stress response may be partly attributed to the ability of the extracts to inhibit the binding and conversion of natural steroid substrates by cytochrome P450 17α-hydroxylase/ 17, 20 lyase (CYP17) and cytochrome P450 21-hydroxylase (CYP21), respectively. Although it was shown that aqueous and methanol *S. frutescens* extracts inhibited the binding of progesterone (PROG) to the microsomal P450 enzymes, the inhibition of binding of pregnenolone (PREG) was negligible. In addition, the metabolism of PROG was inhibited to a significantly greater extent than the metabolism of PREG by ovine CYP17 in COS1 cells. A triterpenoid fraction obtained from *S. frutescens* inhibited the binding of both PROG and PREG to the microsomal P450 enzymes by binding directly to the heme iron in the active pocket of these enzymes. Canavanine, GABA and pinitol did not inhibit the binding of PROG and PREG to the microsomal P450 enzymes [Prevoo *et al* (2004)].

Corticometric effects have been suggested to be linked to the presence of sutherlandiosides [Van Wyk & Wink (2004)]. The sutherlandiosides are cycloartane glycosides with structures similar to that of the steroid substrates of steroidogenic cytochrome P450 enzymes [Fu *et al* (2008)]. Madgula *et al* investigated several properties of sutherlandioside B and showed that sutherlandioside B inhibits CYP3A4 significantly, while this compound had no effect on CYP2D6. In addition, the compound

was not metabolized in liver microsomes and did not exhibit a strong binding affinity for plasma proteins [Madgula *et al* (2008)].

Another compound present in *S. frutescens*, with possible applications in the treatment of depression, is GABA. The presence of GABA in *S. frutescens* could warrant the use of this plant to treat psychiatric disorders, although this has not yet been confirmed through scientific data.

3.5 Conclusion

Although it was originally thought that six different species of *Sutherlandia* existed, it is currently accepted that there are only two: *S. frutescens* and *S. tomentosa*. In addition, it is also accepted that the species belong to the *Lessertia* genus as there is very little genetic variation between the two species.

The medicinal properties of *S. frutescens* have been exploited by the indigenous people of Southern Africa as is evident by its application in depression, cancer, diabetes, anxiety, inflammation, stomach problems and muscle wasting in AIDS patients. Although several compounds have been identified in extracts of *S. frutescens*, some compounds may contribute towards specific bioactivities. It was demonstrated that the presence of pinitol may contribute to anti-diabetic and anti-inflammatory activities, canavanine may have anti-cancer and anti-viral activities and GABA may have anti-anxiety and anti-depression activities. In addition, it has been suggested that the corticometric activity of *S. frutescens* can be attributed to the presence of sutherlandiosides, but the latter has not yet been confirmed. Although sutherlandioside B has been shown to interact with CYP3A4, no such interaction was observed for CYP2D6. This may imply that, although cytochrome P450 enzymes are homologous, this compound may be selective towards different cytochrome P450 enzymes suggesting that other compounds may also be selective towards the enzymes. Although the data suggests that *S. frutescens* extracts do have the potential to treat the proposed ailments, clinical evidence is lacking.

The safety of the use of *S. frutescens* leaf extracts has been investigated and it was demonstrated that 800 mg/day is a safe dosage with no adverse side effects. No studies have yet determined the effects of *S. frutescens* on pregnant or lactating women.

The accumulated preclinical data assesses the value of the use of *S. frutescens* leaf extracts for the abovementioned ailments and has shown overwhelming positive effects. The possibility of synergistic actions between compounds has been mentioned previously and is an area of interest that needs further investigation [Tai *et al* (2004)]. The next step in the research of *S. frutescens* may include identifying the compounds responsible for the bioactivity of extracts of *S. frutescens* and possible synergistic actions between these compounds. In addition, the risks and benefits of the use of *S. frutescens* as a traditional medicine may be investigated through clinical trials. Controlled clinical trials may provide insight into the benefits of using *S. frutescens* for treating chronic diseases.

The majority of the ailments traditionally treated with *S. frutescens* appear to be linked with elevated plasma glucocorticoid levels, especially stress and diabetes. Cortisol, the major glucocorticoid in humans, is synthesized in the adrenal gland by the steroidogenic cytochrome P450 enzymes, thus making these enzymes a possible target for the inhibitory effect of *S. frutescens* on cortisol production. The cytochrome P450 enzymes and their role in the production of cortisol are discussed in the following chapter.

Chapter 4

Adrenal steroidogenesis

All living organisms have the ability to regulate their internal environment by adjusting to different physiological processes. This intrinsic ability is commonly referred to as homeostasis and the regulation thereof is essential for life. When this delicate internal balance is disturbed by a stimulus, be it physical or psychological, it results in a stress response in the organism. The term stress was first used by Hans Selye to describe the reaction of laboratory animals to harmful stimuli. He proposed that the stress response could be explained in terms of the general adaption syndrome. This syndrome is broken down into 3 different stages: (1) alarm – the source of stress is identified and the body responds by initiating the fight or flight response which entails the activation of the HPA axis resulting in the secretion of the catecholamines and of cortisol, (2) resistance – the body needs to cope with the persisting stressor, however, the response cannot be sustained indefinitely and resources are depleted and (3) exhaustion – the body's resources are depleted and the normal functions cannot be sustained [Selye (1956)]. If the organism remains in stage three for extended periods of time, long-term damage could occur which could lead to permanently damaged adrenal glands and an impaired immune system. Peptic ulcers, depression, diabetes, an impaired digestive system and several cardiovascular ailments have also been associated with long-term exposure to a stressor [de Kloet *et al* (1998)]. The adverse effects that are initiated by long-term exposure to a stressor have been linked to the consequential elevation of plasma cortisol concentrations [Chrousos & Gold (1992)].

The cytochrome P450 (P450) enzymes and 3β-hydroxysteroid dehydrogenase (3βHSD) are responsible for the biosynthesis of cortisol in the adrenal cortex [Omura *et al* (1993)]. All adrenal steroids are synthesized from cholesterol by consequential hydroxylation reactions involving P450 enzymes which are regulated by the HPA axis. During the normal stress response, the HPA axis is stimulated and the secretion of ACTH induces the production of cortisol. Cortisol in turn exerts a negative feedback inhibition on the HPA axis to return the plasma cortisol levels to normal [Payne & Hales (2004)]. A

dysfunctioning HPA axis leads to elevated plasma cortisol levels which lead to many adverse effects, such as osteoporosis, cardiac disease, depression, anxiety and diabetes. Therefore, adrenal steroidogenesis plays an important role in maintaining normal cortisol levels and maintaining a normal stress response.

4.1 Adrenal Gland

4.1.1 Anatomy

The adrenal glands are located bilaterally above each kidney and can clearly be divided into an inner medulla and an outer cortex. This gland is unique because it consists of two types of endocrine tissues. The adrenal medulla (inner) consists of neurological tissue and the adrenal cortex (outer) consists of glandular tissue. The zones of the adrenal gland are also functionally different and produce different types of hormones. The adrenal medulla is the site of catecholamine, epinephrine and norepinephrine, production and the adrenal cortex is the site of glucocorticoid, mineralocorticoid and androgen production [Porterfield (2001)].

The adrenal cortex comprises most of the adrenal gland and is the site of adrenal steroid biosynthesis. During gestation, the fetal gland is formed in the first 8 weeks and is actively involved in the biosynthesis of steroids. At this time the majority of the fetal adrenal cortex consists of the fetal zone and the remaining zone is referred to as the definitive zone. The fetal adrenal also plays a major role in the synthesis of placental steroids. The adrenal glands are at their largest at birth and decrease in size during maturation due to the loss of the fetal zone. The definitive zone of the nonfetal adrenal cortex differentiates into three layers: zona glomerulosa, zona reticularis and zona fasciculate (Figure 4.1) [Ishimura & Fujita (1997); Pelletier *et al* (2001)]. The zona glomerulosa is the outer most layer of the adrenal cortex and the enzymes required for mineralocorticoid production are expressed in this zone. Neither androgens nor cortisol are synthesized in this zone as CYP17 is not expressed. The zona glomerulosa is mostly regulated by angiotensin II which stimulates its growth, as well as aldosterone production [Porterfield (2001)].

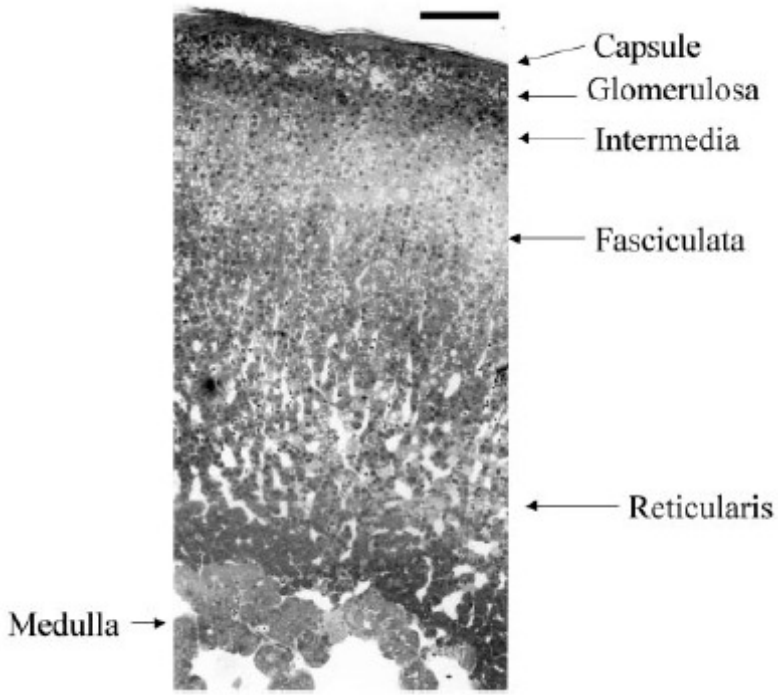


Figure 4.1: Zones of the adrenal gland [Vinson (2003)].

The zona fasciculate and the zona glomerulosa are primarily associated with the production of glucocorticoids while the zona reticularis produces androgens. Aldosterone cannot be synthesized in the zona fasciculata or zona reticularis since the enzyme responsible for the synthesis of aldosterone is expressed only in cells of the zona glomerulosa. Adrenocorticotrophic hormone (ACTH) is responsible for regulating growth of these zones as well as the steroid biosynthesis in these zones [Hinson & Raven (1996)].

4.1.2 Adrenal steroidogenesis

The adrenal cortex is the site of biosynthesis of various steroids which are essential in sustaining life. The most important of these corticosteroids in most mammalian species are the glucocorticoids, cortisol and corticosterone as well as the mineralocorticoid, aldosterone. The steroid that is synthesized at the highest concentration in the adrenal cortex is the androgen, dehydroepiandrosterone sulphate (DHEAS) [Miller & Tyrell (1995); Miller (1988)]. Although androgen precursors are synthesized in the adrenal

cortex, they are not relevant to this study and will therefore not be discussed in detail. In some rodents, there is very little androgen and no cortisol production in the adrenal cortex because of the lack of the 17α-hydroxylase activity. The major glucocorticoid in these animals is corticosterone [Hinson & Raven (1996)].

P450 enzymes and 3βHSD synthesize adrenal steroids from cholesterol by consequential hydroxylation reactions. The adrenal steroidogenesis pathway is shown in Figure 4.2. Cholesterol, which is supplied to the human body through diet and is synthesized in the liver, can be derived from two other sources: synthesis from acetate in the adrenal gland or from the blood plasma through receptor mediated uptake. Although the amount of cholesterol obtained from these different sources varies, in the human and rat adrenal approximately 80 % of the cholesterol used as steroid precursor is taken up from lipoproteins in the blood [Payne & Hales (2004)]. Cholesterol in humans is usually associated with low density lipoproteins (LDL) and in the rat cholesterol is predominantly associated with high density lipoproteins (HDL). The majority of cholesterol is stored as an esterified product that can be quickly accessed in the case of acute stimulation of adrenal steroidogenesis [Vinson *et al* (1992); Hinson & Raven (1996)].

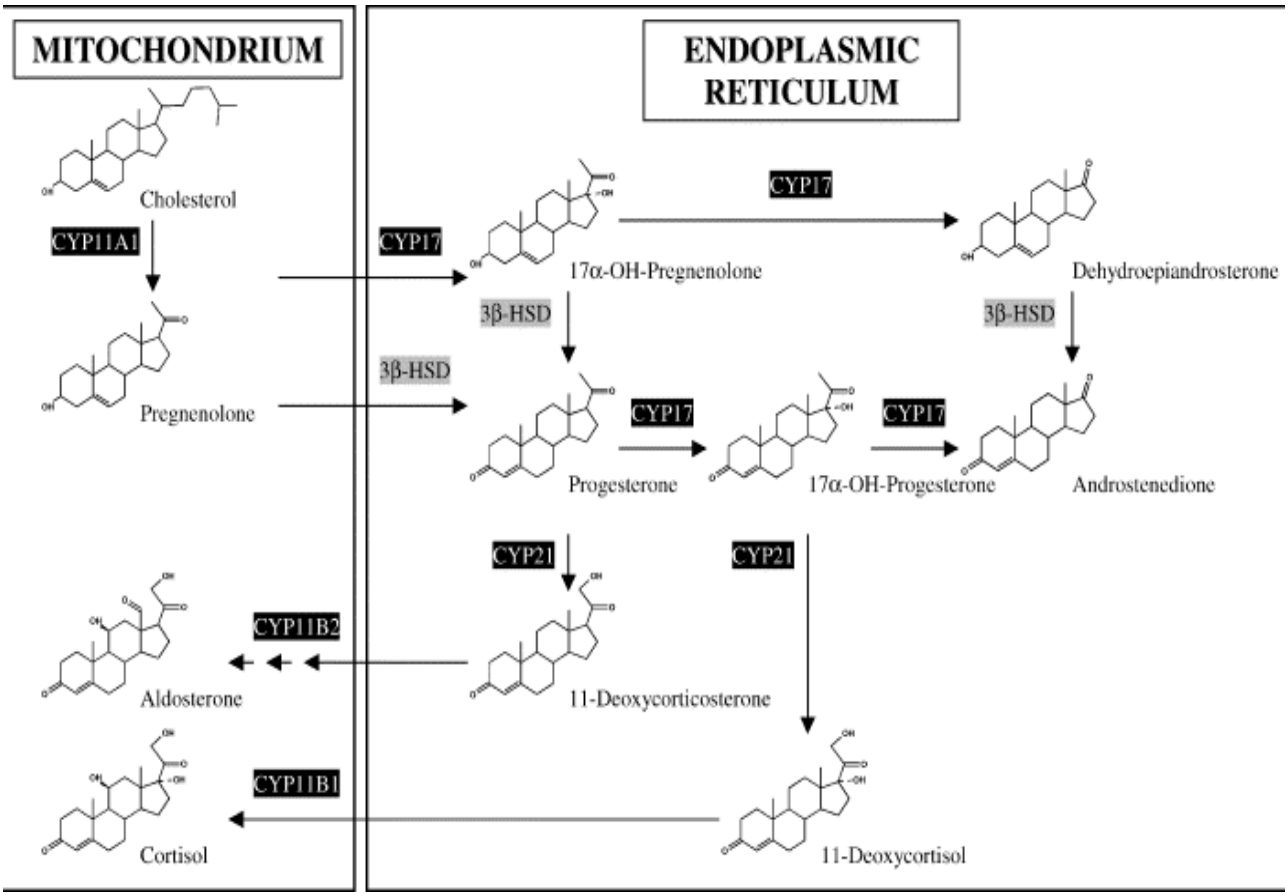


Figure 4.2: Steroidogenesis in adrenal glands. The individual enzymes are highlighted [reproduced from Lisurek and Bernhardt (2004)].

4.1.3 The properties of cytochrome P450 enzymes

It is widely accepted that P450 genes are represented by the abbreviation *CYP*. These abbreviations are followed by an Arabic number that represents the P450 family and a letter designating the subfamily [Nelson *et al* (1996)]. If more than one subfamily exits, the letter is followed by an Arabic number designating the individual gene, e.g. CYP11B1 and CYP11B2.

Although the P450 enzymes have less that a 20 % sequence identity throughout the entire gene superfamily, these enzymes differ surprisingly little regarding their general topology and fold (Figure 4.3) [Hasemann *et al* (1995)]. The structural core of P450 enzymes consists of four helices of which three are parallel (D, L and I in Figure 4.3) and the fourth is anti-parallel (E in Figure 4.3) [Presnell & Cohen (1989)]. The prosthetic heme

group is found between helix L (proximal) and helix I (distal), bound to the contiguous Cys-heme-ligand loop at Cys 357. Surrounding the latter is the signature P450 amino acid sequence, FXXGx(H/R)XCXG, which is highly conserved in cytochrome P450 enzymes.

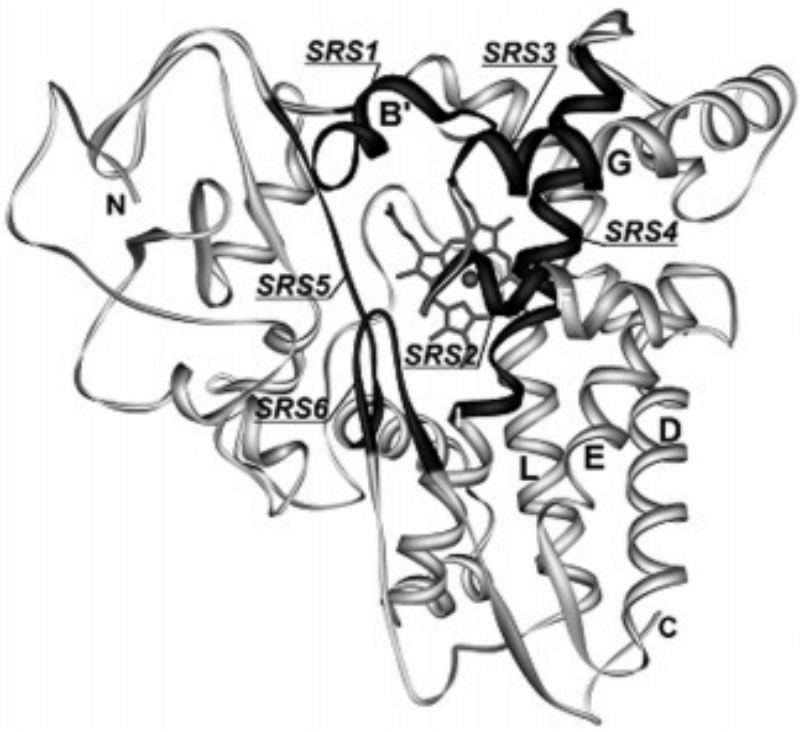


Figure 4.3: Ribbon representation of the typical fold of a cytochrome P450 enzyme. The parallel and antiparallel helices are labeled D, E, L and I. The substrate recognition sequences (SRS) are labeled and printed in black [Hasemann *et al* (1995)].

Helix I consisting of 34 amino acid residues and runs through the middle of the molecule, while helix L is found along the surface. The inner hydrophobic region of the molecule contains the heme skeleton and Arg 112, His 355, Glu108, Arg 299 and Asp 297, which are dissociable amino acids, surround the propionate side chains. The propionate groups interact with these amino acids through atypical carboxyl-carboxyl-hydrogen bonds and normal hydrogen bonds. Stabilization of the heme thus occurs through heme-propionate hydrogen bonds, a heme thiolate bond as well as hydrophobic interactions with the distal helix [Omura *et al* (1993)].

In spite of a highly conserved topology and fold, the P450 enzymes are able to bind to significantly different substrates with differing specificity. Although many P450 enzymes

are highly specific towards their substrates, P450 enzymes such as CYP3A4, which is expressed in the liver, are able to metabolize approximately 50 % of all pharmaceuticals currently marketed [Pylypenko *et al* (2003); Zerbe *et al* (2002)]. Substrate recognition and binding is ensured by the substrate recognition sequences (SRS) in which the sequences are highly variable. The six SRS are: SRS1 in the B-helix, SRS2 and 3 found in the G and F helices, SRS4 in helix I, SRS5 in the β4 hairpin motif and SRS6 in the K helix. SRS5 and SRS6 line the inside of the P450 active site (Figure 4.3) [Gotoh (1992)]. The degree of substrate specificity of P450 enzymes is largely dependent on the SRS. Point mutations within these SRS can drastically effect a P450 enzyme's affinity for its substrate and the binding thereof. These SRS are considered to be accommodating protein regions that move to support substrate binding by an induced fit mechanism which initiates the catalytic cycle [Pylypenko & Schlichting (2004)].

4.1.3.1 Catalytic cycle of cytochrome P450 enzymes

The P450 enzymes share a common catalytic cycle which depicts the roles of the protein, iron and oxygen molecules in reactions catalyzed in by these enzymes (Figure 4.4). These reactions are initiated when substrate binds to the P450 enzymes as shown in reaction 1. The substrate binding induces a conformational change in the enzyme and the redox potential of the heme is increased from approximately -300 mV to -170 mV, thus reducing the enzyme-substrate complex [Sligar *et al* (1976)]. Electrons are transferred to P450 enzymes by specific electron carriers only when it has bound a substrate.

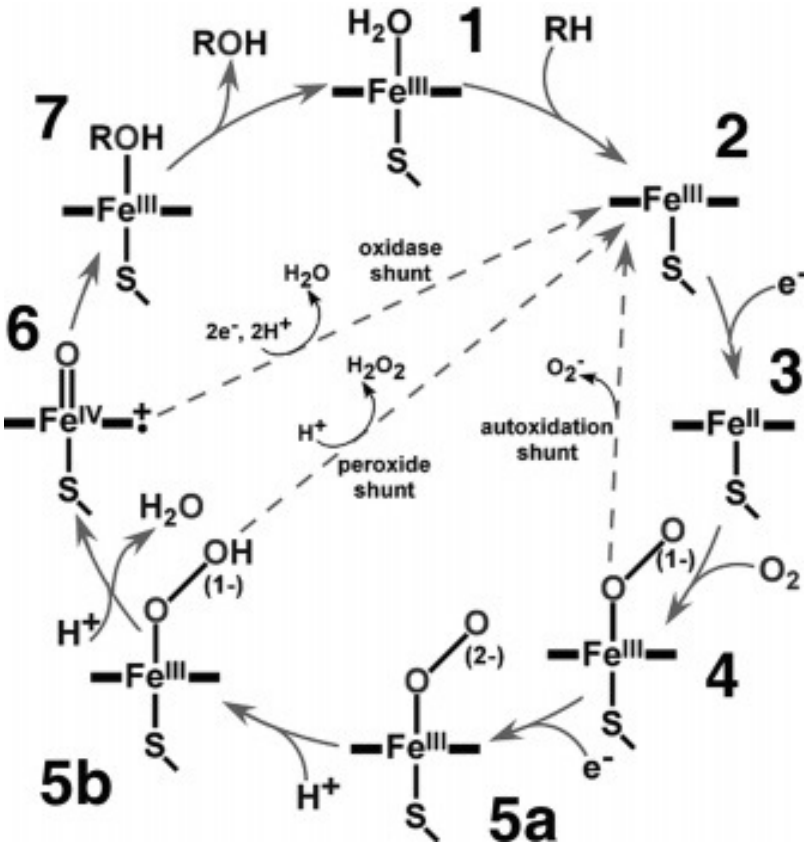


Figure 4.4: Catalytic cycle of cytochrome P450 enzymes [Denisov (2005)].

The free P450 enzyme (RH-Fe^{3+}) has a very low affinity for oxygen molecules, however, when it is reduced (RH-Fe^{2+}) its affinity increases. One oxygen molecule will bind to the reduced heme iron at the 6th axial position of the heme. The bound electron immediately moves to the bound oxygen molecule which results in a very unstable oxygenated P450 enzyme (RH-Fe^{3+}-O_2^-) [Sligar *et al* (1974)]. The second electron is now transferred to the enzyme complex from the electron carrier to produce RH-Fe^{2+}-O_2^-. Some microsomal P450 enzymes also have the potential to obtain the second electron from cytochrome b_5. The O_2^{-2} at the heme reacts with the protons (reaction 5) releasing H_2O from its active centre, resulting in an activated oxygen atom. This activated oxygen atom reacts with the substrate molecule in the enzyme complex and the product is released from the enzyme. The P450 enzyme is now in its original conformation and oxidation state. This reaction takes a mere 1-10 seconds in membrane bound P450 enzymes, but faster reaction times

have been documented [Bonfils *et al* (1979)]. The overall reaction can be represented as Equation 4.1:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+ \quad \text{(Eq. 4.1)}$$

Spectral investigations in liver microsomes have shown that the binding affinity of the P450 enzyme for its substrate is affected by the substrate's hydrophobic characteristics. The enzyme's affinity for the substrate increases with an increase in hydrophobicity of that substrate. This hydrophobic interaction is caused by the hydrophobic amino acid residues that lie within the substrate binding site of P450 enzymes with the heme also providing a hydrophobic plane. The substrate binding pockets of P450 enzymes undergo conformational changes upon substrate binding which ensures that the substrate is in the correct orientation regarding the activated oxygen molecule. The latter determines the regio- and stereospecificity of the P450 enzymes [Perkonen & Viano (1975)].

4.1.3.1.1 Spectral properties

P450 enzymes were first noticed as an independent pigment with an unusual carbon monoxide binding spectrum. The discovery was made when researchers were investigating the oxidation-reduction kinetics of microsome- bound cytochrome b_5. They observed a broad absorption band at 450 nm, after saturating the microsomal suspension with carbon monoxide. This could not be attributed to cytochrome b_5, since carbon monoxide does not affect the α and β bands of this cytochrome. The peculiar ability of the pigment to bind to carbon monoxide suggested that this pigment had a heavy metal atom in its chromophore, but its spectrum showed no similarity to the known hemoproteins and coloured metalloproteins [Garfinkel (1958); Klingenberg (1958)].

In the 1960's Omura and Sato deduced that the pigment was a new b-type cytochrome and named it P450 [Omura & Sato (1964)]. Their first clue to the hemoprotein nature of this pigment was the difference spectrum that occurred when the pigment was reduced with ethyl isocyanide. The resulting spectra showed intense bands in the Soret area and additional α and β bands. They also observed that when microsomes were treated with detergents the pigment was not entirely destroyed, but quantitatively converted to a form

that maintained its ability to bind to carbon monoxide in its reduced state and remained spectrally distinct. This was also observed by other researchers [Klingenberg (1958)]. The solubilized pigment did, however, differ from the original pigment in terms of its carbon monoxide-difference spectrum. The original pigment showed a peak at 450 nm, whereas the solubilized pigment showed a peak at 420 nm with clear α and β bands was subsequently referred to as P420. This provided evidence of the hemoprotein nature of the pigment. In view of the fact that a quantitative conversion of the P450 form to the P420 form took place when microsomes were treated with either detergents or a phospholipase solution, the membrane bound form, P450, also had to be a hemoprotein. From this data Omura and Sato could calculate an extinction coefficient value of 91 $cm^{-1}mM^{-1}$ for the carbon monoxide reduced-difference spectrum of reduced cytochrome P450 for 420-490 nm [Omura & Sato (1964)]. The extinction coefficient value was later confirmed by using purified cytochrome P450 preparations [Ryan *et al* (1975)]. The extinction coefficient value enabled the determination of the P450 concentration of microsomes and has subsequently been used to determine the P450 concentration isolated from many other tissues.

Ultra violet (UV) spectrometry can be used to identify the different intermediates in the catalytic cycle of P450 enzymes (Figure 4.4). The substrate free enzyme has a low spin state and has been documented to absorb maximally at 417-420 nm. As mentioned previously, substrate binding induces a conformational change in the enzyme and also changes the spin state thereof, from a low spin state to a high spin state. The high spin state substrate-bound enzyme absorbs maximally at 391 nm. When the enzyme substrate complex is reduced, the absorption maximum shifts to 408 nm and after binding to molecular oxygen, the maximal absorption shifts to 418 nm. These different spectra are due to the change in the energy levels of the heme locus. The latter is affected by the environment surrounding the heme locus, the bound substrate or inhibitor, and the hydrophobic nature of the heme ligand [Kominami & Takemori (1982); Lewis (1996)]. Thus, the high and low spin states of the enzyme are attributed to the characteristics of the heme locus [Segall (1997)].

Considering the above, it became clear that the binding of a substrate to a P450 enzyme will induce difference spectra. There are, however, documented cases of compounds that are not substrates for these enzymes that may induce such difference spectra. Type I, type II and reverse type I (modified type I), spectra have been observed and these spectra (Figure 4.5) are dependent on the ligand bound in the active site [Lewis (1996); Lewis (2001)].

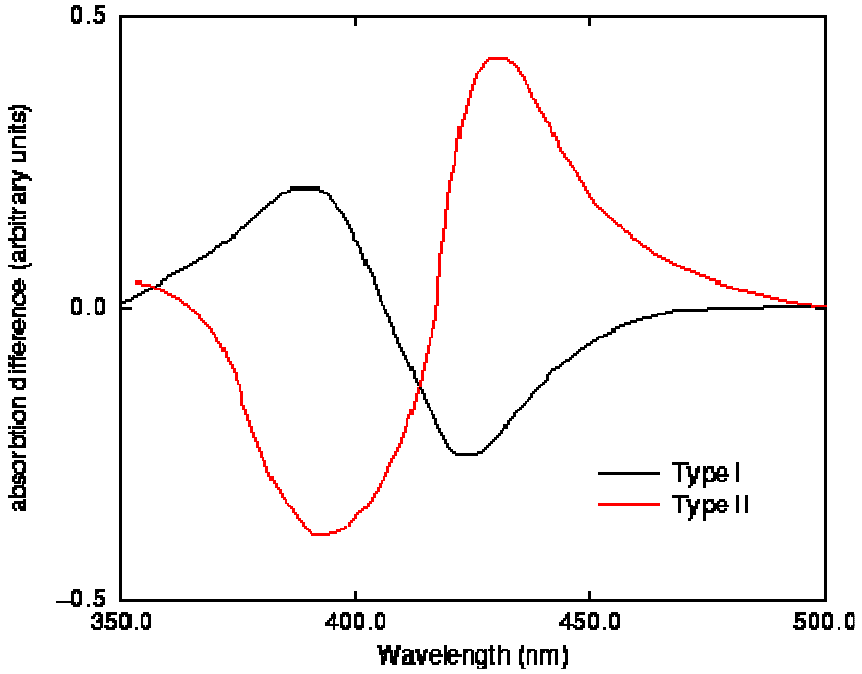


Figure 4.5: Difference spectra observed by UV spectrometry when an inhibitor or substrate is added to the reaction mixture. The black and red lines represent the type I and type II spectra, respectively [Segall (1997)].

Type I difference spectra display a peak maximum at 390 nm and a peak minimum at 420 nm. These spectra graphically represent the interaction between the enzyme and its substrate. It has been shown that high spin state P450 enzymes absorb between 385-394 nm and low spin state enzymes absorb between 417-420 nm. The reaction that takes place involves the displacement of the iron from the ring plane towards the thiolate sulphur, with a type I substrate typically shifting the iron spin equilibrium from a low spin state (peak minimum at 417-420 nm) to a high spin state (385-394 nm) [Sligar *et al* (1976); Hall & Yanagibashi (1986); Lewis (1996); Lewis (2001)]. The change in the spin state of

the iron occurs when a water molecule is displaced, resulting in the iron shifting from the planar porphyrin towards the thiolate ligand. Most P450 substrates are type I substrates that induce a type I spectrum by inducing a high spin state in the enzyme. The spectra can be used to calculate the percentages of the high and low spin state enzymes which can be interpreted as the amount of bound and unbound enzyme [Omura *et al* (1993)].

Type II difference spectra exhibit an absorption maximum between 425-435 nm and an absorption minimum between 390-405 nm and are usually induced by P450 inhibitors. This type of difference spectrum can be observed by adding nitrogen containing compounds, such as amino compounds and imidazole, to the enzyme preparation. Crystallography studies have shown that these compounds bind to the 6^{th} axial position of the heme locus [Omura *et al* (1993)]. Other compounds that have also been documented to induce such a spectrum are compounds containing sulphur and oxygen atoms, as well as unbound electrons that are able to bind to the heme locus [Segall (1997); Omura *et al* (1993)].

Reverse type I spectra have an absorption minimum between 365-410 nm and an absorption maximum between 409-445 nm. These substrates appear not to bind to the heme, but to a different location in the heme pocket. Substrates that induce a reverse type I spectrum are usually hydrophobic molecules which displace the water molecule by hydrophobic interaction when substrate binding occurs.

It would appear that most P450 enzymes display competitive inhibition properties and Michealis-Menton saturation kinetics when considering the kinetics of substrate binding [Omura *et al* (1993)]. Hlavica and Lewis did, however, show that non-hyperbolic kinetics is displayed by certain isozymes [Hlavica & Lewis (2001)]. It has been suggested that an allosteric effect may be the cause of the sigmoidal pattern observed in velocity versus substrate concentration curves. In other words, the second substrate will bind with increased affinity when compared to the first substrate or, the product is generated faster when two substrates are bound to the enzyme compared to when only one substrate is bound [Omura *et al* (1993)].

4.1.4 Mechanisms of inhibition of cytochrome P450 enzymes

P450 enzymes are inhibited though reversible and irreversible mechanisms. Non-covalent interactions with amino acid residues in the active site or the heme group usually display characteristics associated with reversible inhibition. Inhibitors that modify the heme group or the protein itself display irreversible inhibition characteristics. Inhibitors that have the ability to convert a chemically inert moiety to a reactive moiety through the catalytic action of the enzyme are considered to be of great clinical significance. These "activated" reactive moieties should ideally react within the catalytic site where it is produced and should not diffuse into the surrounding environment where it could possibly react with other proteins. Several approaches have been used to design such mechanism-based inhibitors [Ruckpaul & Rein (1989)].

Competitive inhibition is the simplest mechanism of inhibition of P450 enzymes. This type of inhibition implies that there is competition between the inhibitor and the substrate for the occupation of the same active site. If the enzyme therefore has a higher binding affinity for the inhibitor than the substrate, the inhibitor will act in a competitive manner. Competitive inhibitors are usually hydrophobic in order to bind to the hydrophobic active sites of the enzymes and/or contain a nitrogen or heteroatom that could interact with the heme iron atom. Synergism between the metal coordination and hydrophobic interactions increases the ability of the inhibitor to bind to the enzyme by preventing "normal" catalysis through occupying the heme site to which the oxygen would bind under normal circumstances [Ruckpaul & Rein (1989)]. Competitive inhibitors appear to be isozyme specific through the fit of the inhibitor in the active site of the target enzyme and the extent to which an identical structure is excluded from the active sites of other P450 enzymes. Another requirement for competitive binding is that the coordinating atom/moiety should be orientated appropriately in relation to the iron atom when the inhibitor is bound in the active pocket. The orientation of the heteroatom is detected either by the formation of a spectrum with the Soret peak at ± 430 nm or in a type II difference spectrum with a trough at 390-410 nm and peak at 425-435 nm [Jefcoate (1978)].

Mechanism-based inactivation occurs through the modification of the active site of the enzyme to interrupt substrate binding, thus no competition exists between the substrate and the inhibitor for the binding site [Ortiz de Montellano & Reich (1984)]. Mechanism-based inhibitors usually inactivate the enzymes by interacting with their heme group or protein. Although other mechanisms for covalent modification, such as amino acid oxidation have been observed, these mechanisms of covalent modification are not as common [Ruckpaul & Rein (1989)].

4.1.4.1 Electron transfer systems in cytochrome P450 enzymes

The P450 enzymes may generally be divided into two classes according to the electron carriers that are utilized. The class I P450 enzymes are found in the mitochondria and utilize adrenodoxin reductase (ADXR), a FAD containing reductase, and adrenodoxin (ADX), a sulphur protein/ferrodoxin, as electron carriers. ADX and ADXR form a complex and it is the flavin moiety in ADXR that is reduced by NADPH. The electron is consequently transferred from ADXR to ADX which initiates the dissociation of the now oxidized ADX from ADXR. The oxidized ADX molecule complexes with the P450 enzyme-substrate complex and the electron can now be utilized by the enzyme for the hydroxylation reaction [Ziegler *et al* (1999)].

Class II P450 enzymes are localized in the endoplasmic reticulum (ER) and utilize cytochrome P450 reductase to transfer electrons from NADPH. The reductase molecule contains an FAD/FMN moiety and is also membrane bound in the ER. It transfers the electrons directly from NADPH to the enzyme. The above mentioned electron transfer systems are represented in Figure 4.6. The reduction and oxidation of the P450 enzymes are regulated by the redox partners. Uncoupling of the dioxide reduction reaction leads to reactive oxygen species being released [Payne and Hales (2004)].

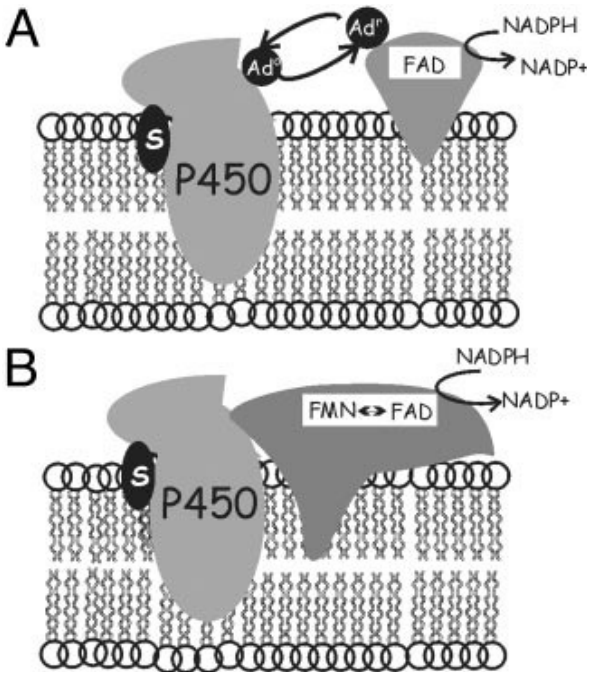


Figure 4.6: Electron transfer systems utilized by (A) mitochondrial cytochrome P450 enzymes and (B) microsomal cytochrome P450 enzymes. FMN: flavin mononucleotide, S: substrate, Ad^0: adrenodoxin, Ad^r: adrenodoxin reductase [Payne and Hales (2004)].

4.1.4.2 Steroidogenic cytochrome P450 enzymes

The cytochrome P450 enzymes involved in adrenal steroidogenesis are associated with either the ER or the mitochondrial membranes of the adrenal cortex. These enzymes are responsible for the biosynthesis of steroids from cholesterol through a series of hydroxylation and cleavage reactions. The cytochrome P450 enzymes will be discussed in reaction order according to Figure 4.2.

4.1.4.2.1 Cytochrome P450 cholesterol side chain cleavage (CYP11A1 or P450scc)

The rate limiting step of steroidogenesis is the delivery of cholesterol to CYP11A1 in the inner mitochondrial membrane. This action is mediated by the steroidogenic acute regulatory (StAR) protein. CYP11A1 catalyses three sequential oxidation reactions of cholesterol by utilizing three molecules of oxygen, the mitochondrial electron transfer system and three molecules of NADPH (Figure 4.7). The hydroxylation at the C22 position is followed by a hydroxylation reaction at C20 to produce 20,22R-

hydroxycholesterol. The latter is cleaved between C22 and C20 and produces pregnenolone, a C21 steroid and isocaproaldehyde. Isocaproaldehyde is oxidized to isocarpoic acid [Boyd & Simpson (1968); Burstein & Gut (1976); Schulster *et al* (1976)].

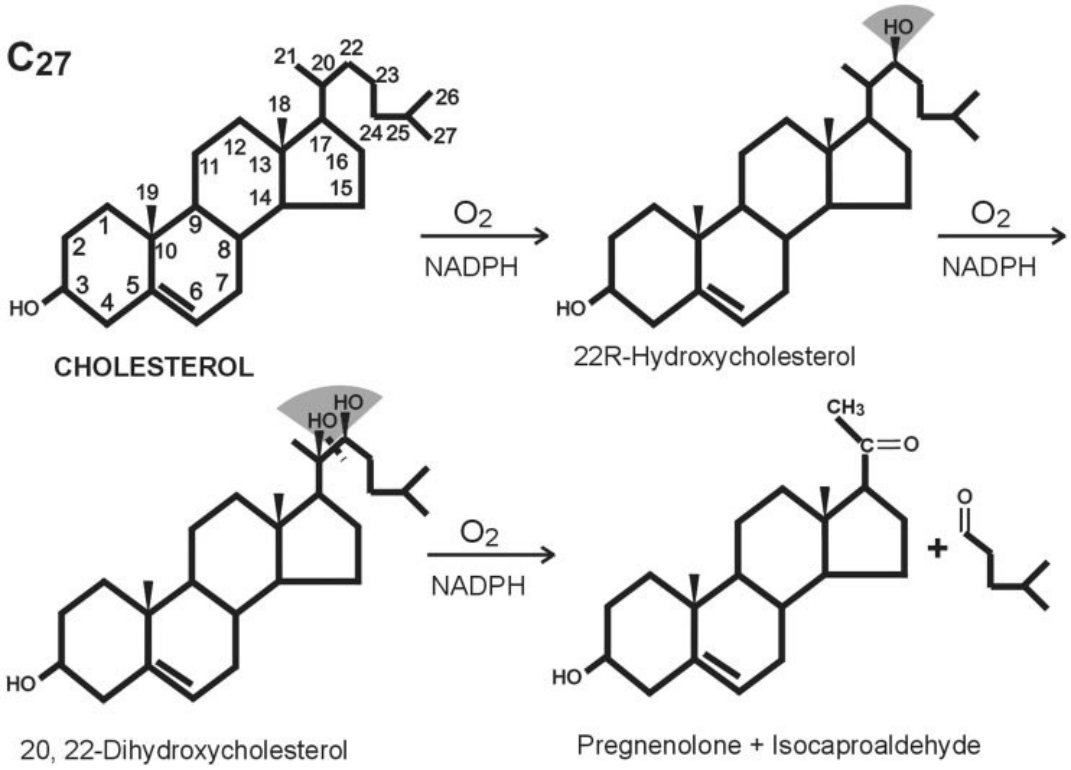


Figure 4.7: Reactions catalyzed by CYP11A1. Three sequential oxidation reactions followed by a cleavage reaction to produce pregnenolone and isocaproaldehyde [Payne & Hales (2004)].

CYP11A1 is a single gene product and the cDNA has been successfully isolated from mice, rats, humans and various other species [Morohashi *et al* (1984); Chung *et al* (1986); Oonk *et al* (1989); Arensburg *et al* (1999)]. The deduced amino acid sequence shows homology of 71 % and higher between species. The human cDNA reading frame encodes a 521 amino acid sequence with a 39 amino acid leader sequence close to the N-terminal that is essential for the translocation of the protein product to the mitochondrial membrane. In addition, the amino acid sequence encodes a heme-binding region close to the carboxyl terminus containing a Cys residue which is conserved throughout the P450 superfamily, as well as one proposed 20 amino acid substrate binding sequence close to

the amino terminus [Okuyama *et al* (1996]. Human *CYP11A1*, which is 20 kb long, is located on chromosome 15q23-q24 [Chung *et* al (1986); Morohashi *et al* (1987)].

Although CYP11A1 is mainly expressed in the adrenal cortex, ovaries, placenta and testis, it has also been detected in peripheral tissues to a much lesser extent. This enzyme is expressed in the zona glomerulosa, zona fasciculata and zona reticularis of the adrenal cortex [Ishimura & Fujita (1997); Pelletier *et al* (2001)]. Investigations into the steroid enzyme expression in fetuses showed that CYP11A1 was expressed most abundantly in the adrenal gland between week 20 and 21 [Voutilainen & Miller (1986)], there after expression of CYP11A1 became essential for survival [Luo *et al* (1994); Hu *et al* (2002)].

4.1.4.2.2 Cytochrome P450 17α-hydroxylase/ 17, 20 lyase

Also known as CYP17 or P450c17, this enzyme utilizes the microsomal electron transfer system and cytochrome P450 oxidoreductase to catalyze mixed function oxidase reactions. The enzyme catalyzes the 17α-hydroxylation of pregnenolone (Δ^5 steroid) and progesterone (Δ^4 steroid), producing are 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, followed by a cleavage reaction that breaks the C17-20 bond producing dehydroepiandrosterone (DHEA) and androstenedione (A4), respectively [Payne & Hales (2004)]. Every step of this reaction requires a molecule of oxygen and a molecule of NADPH. The reactions are shown in Figure 4.8.

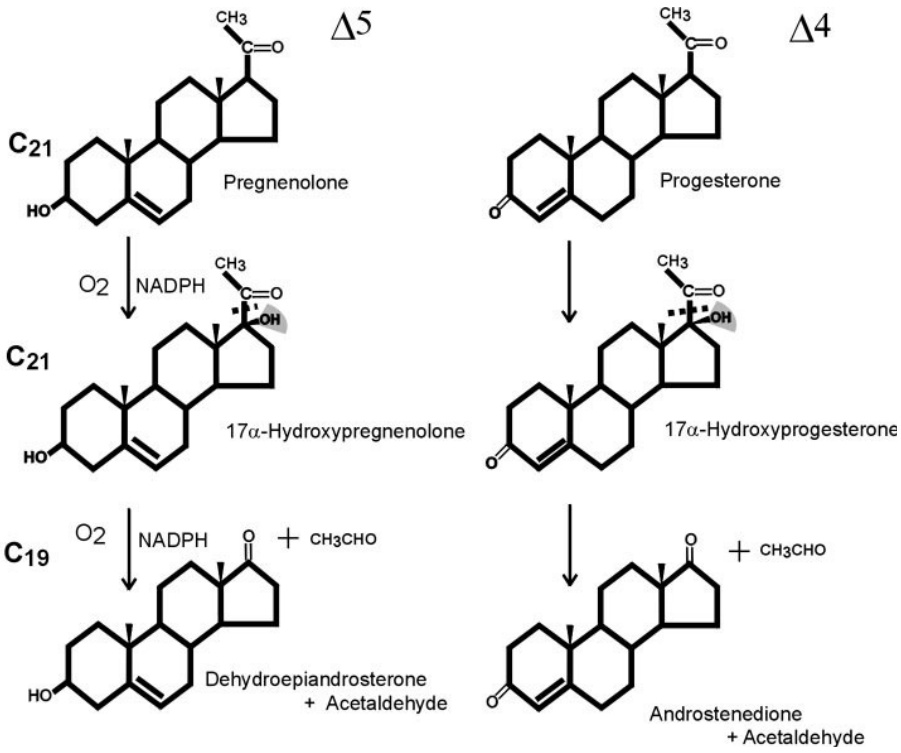


Figure 4.8: Reactions catalyzed by CYP17. The enzymes utilize an oxygen molecule and a NADPH molecule for every step of the reaction [Payne & Hales (2004)].

Although various species of CYP17 will catalyze the hydroxylation and lyase reactions, there are species that differ in their metabolism of the 17α-hydroxy intermediates as substrate for the lyase reaction. Studies have shown that human and bovine CYP17 enzymes have a preference for Δ^5 substrates producing DHEA, whereas the rat enzyme has a preference for the Δ^4 substrates producing A4 [Nakajin *et al* (1981); Zuber *et al* (1986); Swart *et al* (1993); Katagiri *et al* (1995); Brock & Waterman (1999)]. This difference in substrate preference between species is influenced by differences in the amino acid sequence and the presence of an accessory protein, cytochrome b_5. The bovine and human CYP17 enzymes require cytochrome b_5 to promote the lyase activity of the Δ^5 intermediates, but not the Δ^4 intermediates. Cytochrome b_5 increases the V_{max} of CYP17's lyase activity allosterically affecting the C17-oxidoreductase complex [Auchus *et al* (1998)]. Although cytochrome b_5 also stimulates the lyase activity for 17α-hydroxyprogesterone, the increase in activity is insignificant compared to the increase observed in the lyase activity for 17α-hydroxypregnenolone [Brock & Waterman (1999)].

CYP17 is a single gene product and the *Cyp17* gene is approximately 6 kb long containing eight exons and conserved intron-exon boundaries [Youngblood *et al* (1991)]. The CYP17 enzymes have regions of high homology which are common in the P450 gene superfamily — the binding regions which are encoded by the amino acid sequence from 435-455 in human CYP17 and the ozols tridecapeptide sequence which are encoded by the amino acid sequence from 343-372 in most species. The latter is thought to contribute towards substrate specificity [Zuber *et al* (1986)]. Another region that is conserved throughout species is the 296-319 amino acid sequence of the CYP17 enzyme which is thought to be vital for catalyses. Furthermore, Arg^{346} and Arg^{347} have been found to be essential for the catalyses of the lyase reaction in rat and human CYP17, respectively [Kitamura *et al* (1991); Lin *et al* (1994)].

CYP17 has been found in all the typical steroidogenic tissues, but different levels of expression have been observed in the adrenal gland and placenta of different species. In the adrenal glands of humans and bovine, CYP17 is expressed in the zona fasciculata and the zona reticularis, but no expression is observed in the zona glomerulosa [Endoh *et al* (1996); Hyatt *et al* (1983)]. However, in the adrenal glands of the rat and mouse, no CYP17 is expressed and these rodents produce corticosterone instead of cortisol [Perkins & Payne (1988); Brock & Waterman (1999)].

4.1.4.2.3 Cytochrome P450 21-hydroxylase

CYP21 (P450c21) is a microsomal enzyme that catalyzes the hydroxylation of progesterone and 17α-hydroxyprogesterone to produce 11-deoxycorticosterone (DOC) and deoxycortisol, respectively (Figure 4.9). The reaction requires one molecule of oxygen and one molecule of NADPH, and utilizes the microsomal electron transfer system. In the adrenal zona glomerulosa, where no CYP17 is expressed, (PROG) is the substrate for CYP21. CYP17 cannot hydroxylate DOC at C17 and therefore DOC cannot be an intermediate for cortisol production (Figure 4.9) [Payne & Hales (2004)]. Once PROG has been hydroxylated by CYP21 to produce DOC it is committed to the mineralocorticoid pathway.

In humans, there are two species of *CYP21* genes, *CYP21A* and *CYP21B*, of which only *CYP21B* encodes an active enzyme. The human *CYP21A* gene contains a one-base

insertion, a deletion and a transition mutation. The latter is suggested to be responsible for the premature termination of the translation process [White *et al* (1986); Higashi *et al* (1986); Rodrigues *et al* (1987)].

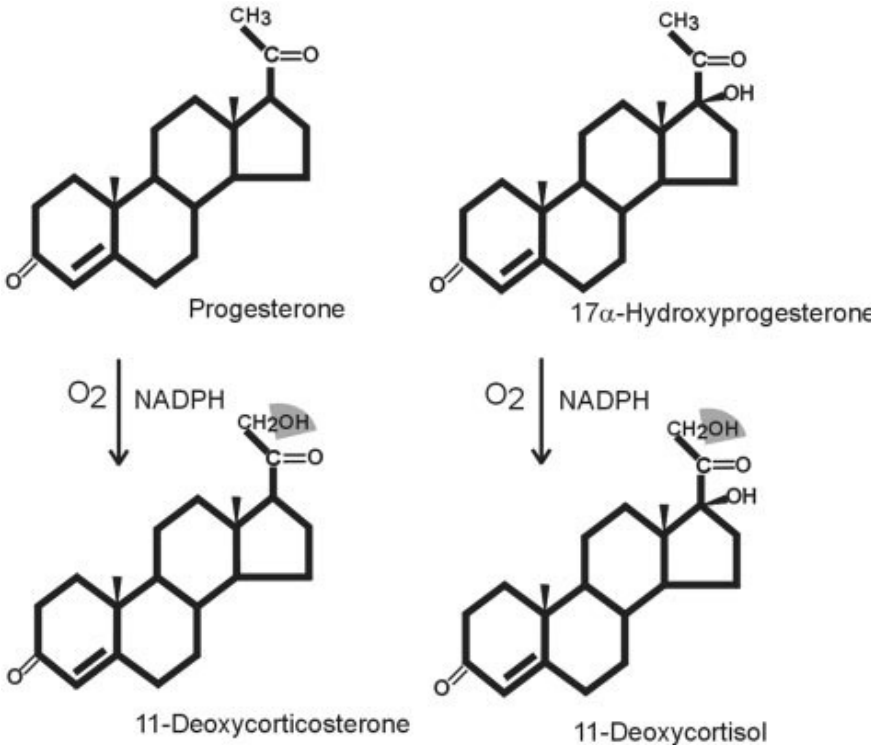


Figure 4.9: Reactions catalyzed by CYP21. The reactions catalyzed by this enzyme are the 21-hydroxylation of progesterone and 17α-hydroxyprogesterone [Payne & Hales (2004)].

The two human CYP21 isoforms are highly homologous in their flanking regions and introns containing 10 exons in total [Higashi *et al* (1986)]. These genes are located on chromosome 6p21.3 and the enzyme has a molecular mass of 56 kDa. The protein product consists of a 495 amino acid sequence [Hanukoglu (1992)].

The expression of CYP21 is limited to the adrenal cortex where it is expressed in all three zones of the adrenal cortex: zona glomerulosa, zona reticularis and zona fasciculate. CYP21 is vital for the production of the mineralocorticoid, aldosterone, and the glucocorticoids, cortisol and corticosterone [Rice *et al* (1990); Wijesuriya *et al* (1999)].

4.1.4.2.4 Cytochrome P450 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2)

CYP11B1 and CYP11B2 are also known as P450c11b1 and P450c11b2, respectively, and are expressed in the inner mitochondrial membrane. In the mitochondria 11-deoxycortisol and DOC are hydroxylated at C11 by CYP11B1 to produce cortisol and corticosterone, respectively (Figure 4.10). This enzyme has the capacity to hydroxylate DOC at C18 to produce 18-hydroxycorticosterone, but it is unable to oxidize the 18-hydroxy group to produce aldosterone. Aldosterone is produced by CYP11B2 from DOC through three sequential reactions that require an oxygen molecule, a NADPH molecule and the mitochondrial electron transfer system for every reaction (Figure 4.11).

It has been suggested that no intermediates are released during the three sequential reactions and that CYP11B2 cannot effectively utilize corticosterone as a substrate to produce aldosterone. This indicates that CYP11B1 does not play a role in aldosterone production and that the three sequential reactions (Figure 4.11) are mediated by CYP11B2 only [Kawamoto *et al* (1992)].

CYP11B1 and CYP11B2 are closely linked and are found on chromosome 8q21-22 in humans. The two genes are oriented in such a way that they are transcribed in the same direction with CYP11B2 on the 5' side of the CYP11B1 gene. These two genes display approximately 95 % sequence identity and 90 % intron identity. Remarkably, the introns of CYP11B1 and CYP11B2 are located in the same positions as the introns of CYP11A1. The binding regions, the aromatic regions and the region responsible for the ozol peptide are almost identical for CYP11A1, CYP11B1 and CYP11B2 [Mornet *et al* (1989)].

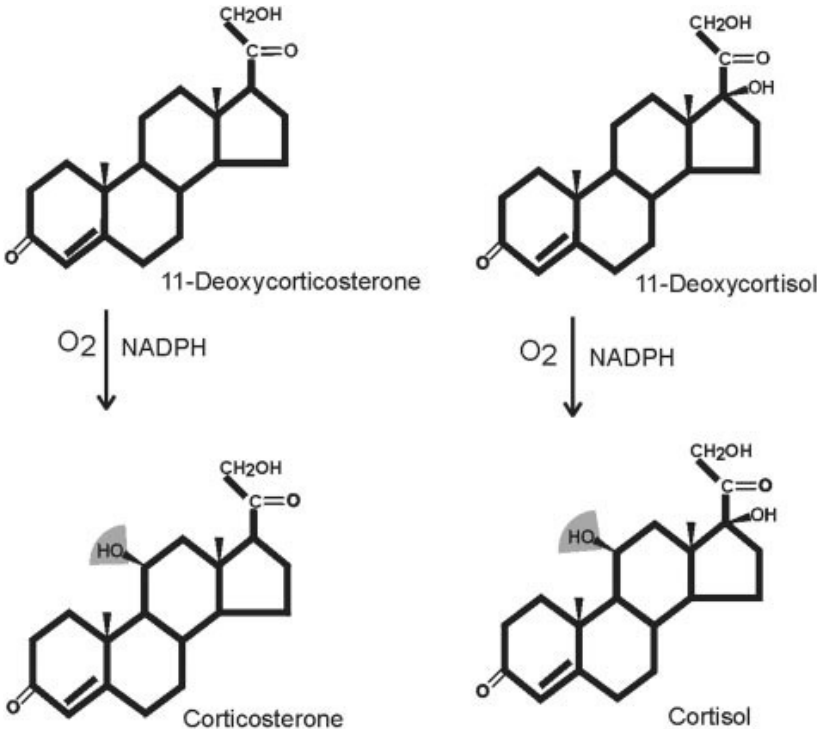


Figure 4.10: Reactions catalyzed by CYP11B1 [Payne &Hales (2004)].

The human CYP11B1 and CYP11B2 amino acid sequences are 93 % identical and are synthesized with a 24 amino acid leader sequence which is cleaved when the protein product is inserted into the mitochondrial membrane to produce the active protein [Mornet *et al* (1989)]. CYP11B1 and CYP11B2 are expressed in the adrenal cortex only. CYP11B2 is expressed only in cells of the zona glomerulosa, whereas CYP11B1 is expressed in the zona fasciculate [Hinson & Raven (1996)].

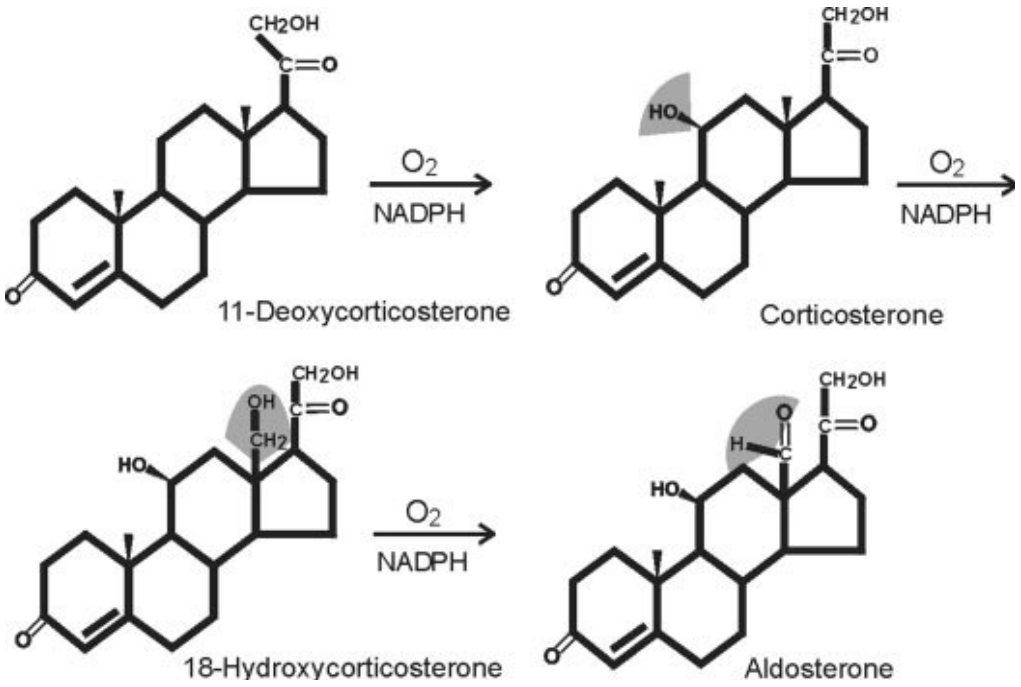


Figure 4.11: Reactions catalyzed by CYP11B2 [Payne & Hales (2004)].

4.1.5 Expression of steroidogenic cytochrome P450 enzymes

Cell-specific expression of P450 enzymes in adrenal glands is determined and regulated by a nuclear factor called steroidogenic factor-1 (SF-1). SF-1 is a nuclear DNA-binding protein which is part of the orphan nuclear receptor family and is known to bind to the AGGTCA sequence. This sequence is located in the proximal promoter region of all P450 genes [Lala *et al* (1992); Morohashi *et al* (1992); Parker & Schimmer (1997)]. Although SF-1 is essential for cell-specific expression of P450 enzymes in adrenal glands, other factors also facilitate cell-specific expression as well as maximizing expression efficiency. For example, the adrenal-specific expression of the human CYP21 gene is regulated by cell-specific elements that are found within the C4B gene that contain three SF-1 sites [Wijesuriya *et al* (1999)].

The synthesis of steroidogenic P450 enzymes in the adrenal gland is stimulated by cAMP through a cascade of reactions. It starts with stimulation by the pituitary peptide hormone, ACTH, which acts through G protein-coupled receptors, activating adenylate cyclase. The latter increases the level of cAMP and stimulates the production of P450 enzymes [Waterman (1994); Waterman & Keeney (1996)]. It appears that during hormonal

stimulation by cAMP, the cAMP response element / cAMP response element binding protein (CRE/CREB) system is not responsible for the increased cAMP level, although there are some exceptions. cAMP response elements are located in various regions in the promoters of the P450 genes. These elements differ in location in different genes as well as in the different species of the same gene. With the exception of CYP17, the expression of all steroidogenic P450 genes is enhanced by increases in cAMP and supplementary factors are required to maintain maximal expression [Parker & Schimmer (1997)]. In contrast to other P450 enzymes, the expression of CYP11B2, which catalyzes the synthesis of aldosterone, is mainly regulated by angiotensin rather than ACTH. Transcription of *CYP11B2* is stimulated by potassium ions and angiotensin II by increasing the intracellular concentration of Ca^{2+} and also by increased cAMP levels. It has been suggested that cAMP and Ca^{2+} act on the same elements in the *CYP11B2* gene promoter, and thus cAMP and Ca^{2+} can increase the transcription of this gene independently. This indicates that the protein kinase C pathway is not required for increased transcription of *CYP11B2*, and even though the *CYP11B2* promoter does contain a SF-1 binding site, SF-1 is also not a requirement for regulating transcription [Bassett *et al* (2002)].

4.1.5.1 Intracellular regulation of steroidogenesis

ACTH and angiotensin II are both peptide hormones which act on plasma membrane receptors. These receptors are part of a superfamily of membrane receptors that have seven transmembrane domains and both have a similar mechanism for signal transduction [Lewis (2001)].

ACTH binds to adrenocortical cell receptors which causes the activation of adenylyl cyclase through the action of G-proteins. The latter increases cAMP production, which activates protein kinase A resulting in the phosphorylation of the target proteins, leading to the stimulation of steroidogenesis. ACTH increases steroid enzyme expression in the long-term, but acute ACTH action leads to increased levels of cholesterol being delivered to CYP11A1 which is the rate limiting step in adrenal steroidogenesis. This is a result of the stimulating effect protein kinase A has on cholesterol ester hydrolase. Another protein that is activated by ACTH is StAR. StAR mediates the transport of cholesterol across the

mitochondrial membrane and in so doing further increases the amount of cholesterol delivered to CYP11A1 [Vinson *et al* (1978)]. Chronic secretion of ACTH up-regulates the transcription of all the adrenal steroidogenic enzymes including adrenodoxin. LDL and HDL receptor synthesis is also up-regulated which leads to an increase in the availability of cholesterol to be taken up by CYP11A1 into the steroidogenic pathway.

Angiotensin II has a different mechanism of action to ACTH. Angiotensin II binds to AT1 receptors, which are plasma membrane receptors, which activate phospholipase C through the action of G-proteins. Phospholipase C hydrolyses phosphatidyl inisitol bisphosphate to produce two second messengers: diacylglycerol and inisitol triphosphate (IP_3). IP_3 causes calcium release from the ER and the free calcium induces a calcium/calmodulin dependant protein kinase. Diacylglycerol on the other hand activates protein kinase C. It would appear that the pathways that are stimulated by the action of angiotensin II are those associated with aldosterone synthesis. Acute angiotensin II secretion causes an over stimulation of the protein kinases which leads to an increase in the conversion of cholesterol to pregnenolone [Muller (1988)].

4.1.6 Regulation of corticosteroid secretion

The secretion of the glucocorticoids is regulated by ACTH as mentioned in section 4.1.5. This small 39 amino acid peptide hormone is secreted by the anterior pituitary gland and produced by the sequential cleavage of proopiomelanocortin (POMC). Although POMC secretion from the pituitary gland is stimulated by corticotrophin releasing hormone (CRH), it is additionally regulated by an endogenous diurnal rhythm, feedback inhibition by cortisol and stress. CRH, which is transported in the blood bound to CRH-binding protein, binds to receptors on the anterior pituitary when secreted into the hypophyseal portal blood and induces POMC transcription through the activation of adenylate cyclase [Giguere *et al* (1982)].

POMC is broken down to smaller proteins in a tissue-specific manner resulting in the secretion of pro-ACTH and β-lipoprotein. Pro-ACTH is cleaved to produce ACTH, an N-terminal peptide and a joining peptide [Seidah & Chretien (1981)]. Although the N-terminal peptide and the joining peptide have unknown functions, it has been suggested

that these proteins have mild steroidogenic activity and may supplement the action of ACTH [Donald (1980)].

Although CRH is the primary stimulus for ACTH secretion, arginine vasopressin (AVP) has also been reported to stimulate secretion. AVP functions by acting on a V1B receptor through which it activates protein kinase C. ACTH secretion does vary throughout the day and the responsiveness of ACTH secretion to stimulation by CRH is influenced by the HPA axis. Although the responsiveness of ACTH secretion increases in patients administered with corticosteroids, it decreases in individuals suffering from Cushing's disease [River & Vale (1983); Watanabe *et al* (1989)]. In addition, proinflammatory cytokines such as tumor necrosis factor α, interleukin-1 and interleukin-6 elevate ACTH secretion directly or by altering the effects of CRH. The latter explains the response of the HPA axis to stimulation by the inflammatory agents and constitutes a significant immune-endocrine interaction.

Physical stressors elevate the secretion of ACTH and cortisol through the action of AVP and CRH, therefore cortisol secretion will increase in reaction to injury, hypoglycemia, exercise and hypotension [Reul *et al* (1998)]. Severe psychological stress also elevates cortisol secretion. However, cortisol secretion is not influenced in individuals suffering from chronic anxiety. Depressed individuals do, however, have elevated cortisol levels [Aguilera (1994); Streeten *et al* (1984)]. Stress, be it physical or psychological, is one of the main stimuli for the activation of the HPA axis, illustrated in Figure 4.12 [Buckingham *et al* (1992); Dallman *et al* (1987)].

Corticotropin affects the adrenal gland in many different ways. Acute ACTH secretion causes increased blood flow to the adrenal gland, increased steroid synthesis and secretion, as well as a decrease in the level of adrenal ascorbic acid [Miller & Tyrell (1995)]. Long-term exposure of ACTH causes the adrenal cortex to grow and increases steroid output [Momoi *et al* (1992)]. If ACTH is secreted excessively, it causes enlargement of the adrenal cortex and cortisol production increases which could lead to diseases such as Cushing's syndrome [Vinson *et al* (1978)]. A specific level of ACTH secretion is thus needed to maintain normal adrenal function. If ACTH is absent, the adrenal gland atrophies and glucocorticoid secretion ceases. This is a side-effect of

prolonged exposure to synthetic glucocorticoids used for the treatment of inflammatory diseases and chronic stress, which results in a loss of the HPA response to normal stress stimuli and is potentially life threatening [McKay & Cidlowski (1999)].

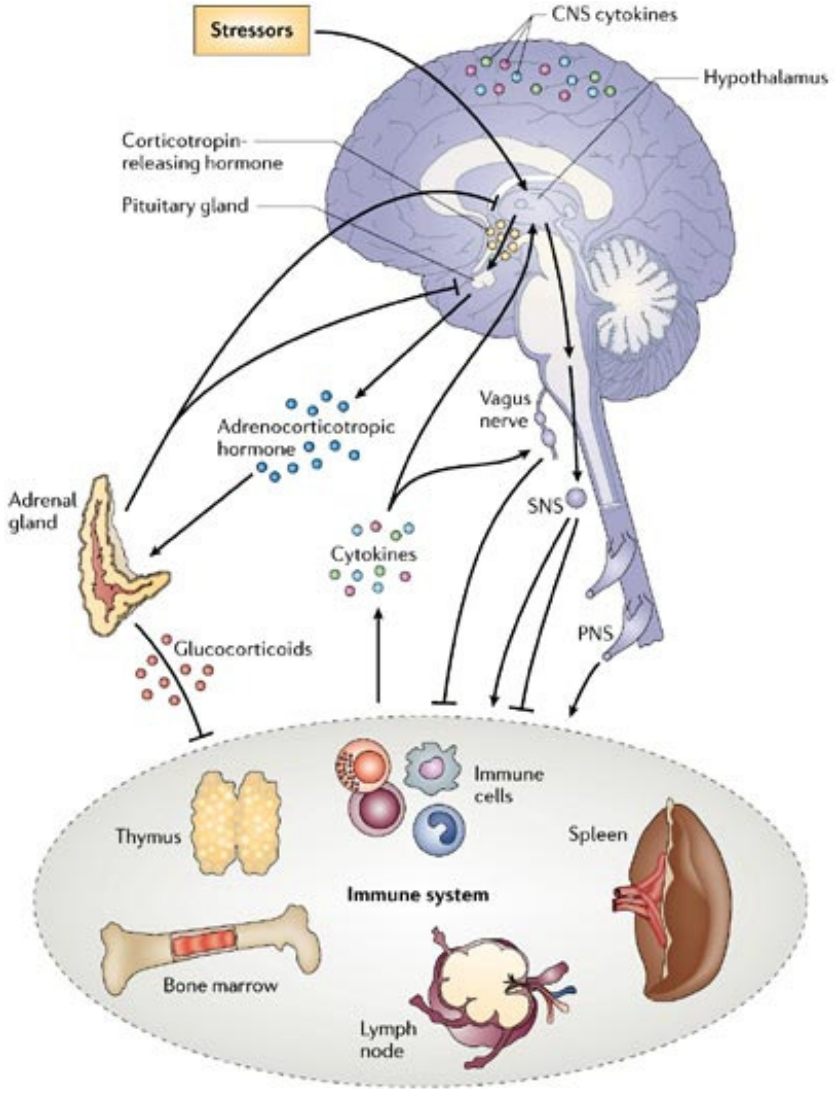


Figure 4.12: Cascade of hormones that regulate the stress response in the HPA axis . [reproduced from Glaser & Kiecolt-Glaser (2005)].

The production of glucocorticoids naturally increases with age. It has been reported that the HPA function is altered with aging in rats and possibly in humans [Seeman & Robbins (1994)]. This altered HPA function results in cortisol levels remaining elevated for longer periods when cortisol secretion is stimulated by a stressor, indicating a dysfunctioning HPA axis. In the adrenals of aged rats, a marked decrease in

corticosterone production per gram of adrenal weight and decreased responsiveness to ACTH was observed by Vinson *et al*. The hypothalamus and the pituitary show increased activity in aged rats. With regard to sex differences, it appears that female rats are more responsive to ACTH secretion, but this could be a feature related to sexual dimorphism observed in the adrenal morphology of these rodents [Vinson *et al* (1978)].

4.1.7 The stress response

Psychological stress induces the secretion of chemical mediators such as serotonin, acetylcholine and norepinephrine (NE) that activate cells in the paraventricular nucleus (PVN), which is located in the hypothalamus. The cells of the PVN produce CRH which induces the corticotrophs in the anterior pituitary to secrete POMC and the locus coeruleus to release NE at sympatic nerve endings [Reul *et al* (1998)]. The activation of the sympatic nervous system (SNS) results in the activation of the chromaffin cells in the adrenal medulla with the consequent release of epinephrine (E). POMC is cleaved to produce ACTH, which in turn stimulates the adrenal cortex to produce corticosteroids as previously mentioned. Although the glucocorticoids, NE and E are the major stress hormones, during acute stress growth hormone (GH) and glucagon are also released and considered to be stress hormones. The stress response also induces the production of angiotensin II in the kidney through stimulating the SNS, which contributes to an elevated heart rate and blood pressure [Dallman & Jones (1973)].

Although CRH is considered to be the major mediator of the stress response, other factors, such as the neuropeptide Substance P (SP), have the ability to act on the SNS and the HPA axis thereby contributing to the stress response [Calogero *et al* (1988); Chen *et al* (1991)].

The stress response may also activate the inflammatory response. It has been suggested that the stress response evolved from the inflammatory response and therefore these two processes are closely linked [Maier & Watkins (1998)]. The coupling of these two responses has obvious survival advantages for animals injured in a fight or flight situation. An inflammatory stimulus could induce the HPA axis to produce CRH and although the stress response has been activated, the physiological modifications that occur could prepare an organism to fight an infection. The corticosteroids initiate the

production of cytokines and other inflammatory agents, therefore contributing towards the initiation of the inflammatory response [Dantzer *et al* (1999)].

4.1.8 Negative feedback inhibition

Glucocorticoids elicit a negative feedback effect on ACTH and CRH secretion. In the anterior pituitary POMC transcription is inhibited and in the hypothalamus AVP and CRH mRNA synthesis and secretion is inhibited by glucocorticoids. The glucocorticoid receptor (GR) is primarily responsible for the feedback inhibition. Jacobson showed that hypersecretion of ACTH and cortisol occurs due to the lack of negative feedback in GR deficient mice [Jacobson (2005)]. This regulatory mechanism may be manipulated with synthetic glucocorticoids in the treatment of chronic inflammation since the negative feedback inhibition is influenced by potency, dose, half-life and duration of glucocorticoid administration. Although glucocorticoids have valuable diagnostic applications, the HPA axis may be suppressed for a certain period after corticosteroid treatment and inadequate adrenocortical function may be expected thereafter.

4.1.9 Corticosteroids

4.1.9.1 The glucocorticoid receptor

Glucocorticoids and mineralocorticoids elicit their effect after these hormones have been taken up from the blood and bound to an intracellular receptor. These receptors are called the glucocorticoid (GR) and mineralocorticoid (MR) receptors respectively. The GR and the MR have a ligand binding domain (C-terminal), a DNA binding sequence and a variable N-terminal region which constitutes the transcriptional activation function (AF-1) domain. The GR structure is shown in Figure 4.13. Both these receptors are encoded by a single gene, but variants have been observed and are referred to as α and β variants [Enrico & Detera-Wadleigh (1991); Zennaro *et al* (1997)]. Steroid binding to GRα induces the dissociation of heat shock proteins, HSP70 and HSP90, which causes the activation of the steroid-receptor complex. The steroid-receptor complex translocates to the cell nucleus and activates or suppresses gene transcription after binding to specialized DNA sequences, known as the glucocorticoid response element, in target genes [Beaton & Sanchez-Pacheco (1996)]. The glucocorticoid response element, a

CGATCAnnnTGTACT sequence, binds to specific loops in the DNA binding domain of the GR, stabilizing the RNA polymerase II complex, thus promoting transcription. It appears as though the GRβ variant has a dominant effect on GRα transactivation through negative regulation [Oakley *et al* (1996)].

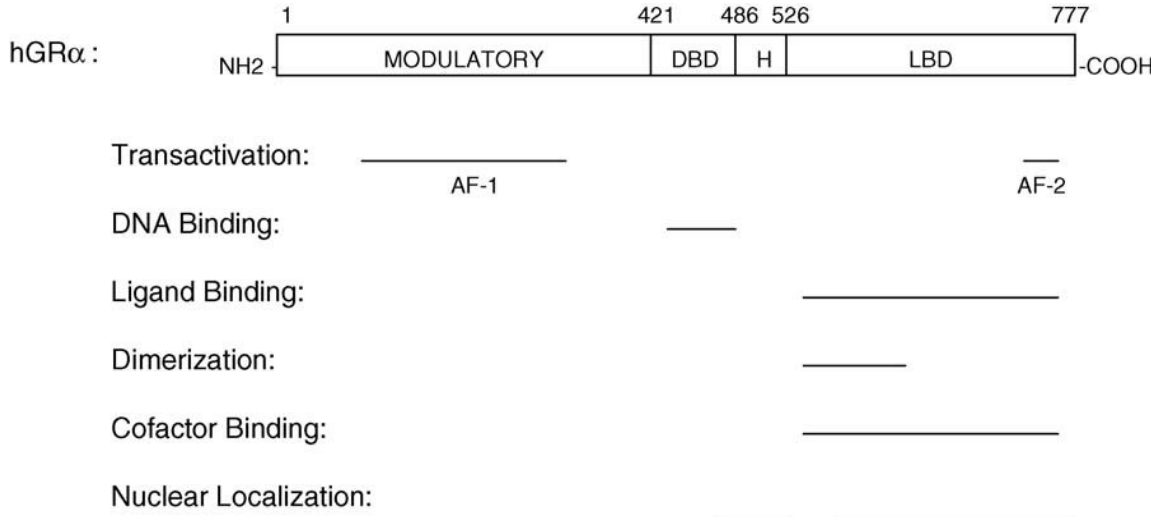


Figure 4.13: Motifs of the human glucocorticoid receptor (hGR). The N-terminal region constitutes the activation function-1 (AF-1), the central region constitutes the DNA binding domain and the C-terminal region constitutes the ligand binding domain and the ligand-dependent activation function (AF-2) [Zhou & Cidlowski (2005)].

The interaction between the GR and certain transcription factors are responsible for inducing the anti-inflammatory effect through glucocorticoid action. Cytokines and phorbol ester induces Activator protein-1 (AP-1) which is a proinflammatory transcription factor consisting of Jun and Fos subunits. If the GR-ligand complex binds to c-jun, it suppresses the interaction with the complex and AP-1. Thus, the transactivation potential of AP-1 and the GR is prevented. Nuclear factor kappa B (NF- κB) is a transcription factor that induces transcription of the genes responsible for the inflammatory response, lymphocyte production and apoptosis and has a similar effect on GR transactivation as AP-1 [McKay & Cidlowski (1999)].

4.1.9.2 Cortisol transport

More than 90 % of cortisol in blood is bound to proteins, with cortisol binding globulin (CBG), synthesized in the liver being the predominant carrier protein. Although CBG has a high affinity for cortisol, this is not the case for most synthetic corticosteroids with the exception of prednisolone. The concentration of CBG in circulation is increased by the presence of estrogens and in individuals suffering from hepatitis, but decreased by the presence of glucocorticoids and in individuals suffering from nephrosis, hyperthyroidism and cirrhosis. The normal concentration of CBG in circulation is approximately 700 nmol/L [Hammond (1990)].

Although abnormalities in CBG synthesis appear to influence the total concentration of cortisol available in circulation, the concentration of the free cortisol still remains normal. Only free cortisol can be taken up into tissues to initiate a biological response. The unbound cortisol excreted from the kidneys, called urinary cortisol, represents 1 % of the cortisol secreted into the bloodstream and is a measure of the total cortisol secretion rate. The urinary cortisol consists of various precursors and intermediates of cortisol metabolism: ±50 % is tetrahydrocortisol (THF), allo-THF and tetrahydrocortisone (THE), 25 % cortolones and cortols, 10 % C19 steroids, 10 % cortolonic, cortolic acids and free steroids which consists of varying concentrations of cortisone, cortisol, THF and THE metabolites.

Although the liver was originally thought to be the major site of cortisol metabolism, studies have shown that the mammalian kidney is also a noteworthy site. The major reaction in the kidney is the inactivation of cortisol by 11β-hydroxysteroid dehydrogenase (11β HSD) to produce cortisone. 11βHSD is also expressed in peripheral tissues and contributes to the regulation of the action of the corticosteroids [Opperman *et al* (1997)].

4.1.9.3 Rhythmic secretion of glucocorticoids

All mammals have a circadian rhythm of glucocorticoid secretion which depends on the animal's phase of activity. The peak of glucocorticoid release occurs in the early morning in diurnal animals and in the early evening in nocturnal animals [Klemcke *et al* (1989); Cheifetz (1971)]. Although shorter pulses of glucocorticoid secretion have been

observed, these pulses are small in comparison to the early morning/evening secretions and have been linked to factors such as food intake [Lincoln *et al* (1982)]. ACTH is secreted, in a rhythmic pattern similar to that of the glucocorticoids, by the corticotroph cells in the pituitary gland. ACTH binds to the type 2 melanocortin receptors located mainly in the zona fasciculate and zona glomerulosa of the adrenal cortex and stimulates the synthesis of corticosteroids [Norris (2007)]. ACTH secretion can also be stimulated by the secretion of AVP, CRH and oxytocin into the bloodstream [Carnes *et al* (1989); Chrousos (1998)]. Although the translation and transcription of CRH appears to be influenced by the circadian rhythm, the secretion of ACTH precedes CRH gene transcription thereby implying that these two processes are loosely coupled [Watts *et al* (2004)]. The control mechanism for circadian glucocorticoid secretion that is discussed above is one of three hypothetical mechanisms (Figure 4.14), and is referred to as the "drive" mechanism since glucocorticoid secretion is driven by the HPA axis.

The circadian secretion of ACTH and glucocorticoids is also controlled by the suprachiasmatic nucleus (SCN) which is located in the anterior hypothalamus. [Klein *et al* (1991)]. The SCN is considered to be responsible for the entrainment of the circadian clock of the adrenal gland through the autonomic nervous system (ANS) and not by influencing the HPA axis. The fact that light information is transmitted via a neural signal to the adrenal gland to promote corticosterone release indicates that autonomic neural control of circadian glucocorticoid secretion exists, as shown in the "entrainment" mechanism in Figure 4.14. This autonomic SCN-adrenal pathway alters the sensitivity of the adrenal gland to ACTH thereby gating the sensitivity of the adrenal gland to stimulation by ACTH shown in Figure 4.14 as the "gating" mechanism. [Buijs *et* al (1999);Ueyama *et* al (1999)].

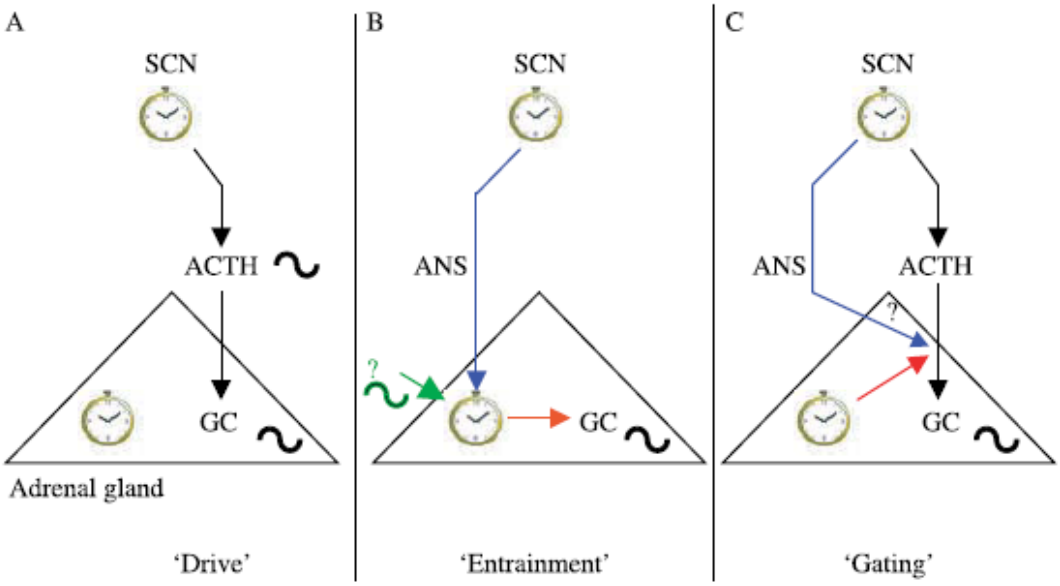


Figure 4.14: Models for the control mechanisms of circadian glucocorticoid secretion. A) Rhythmic changes in the HPA axis alters glucocorticoid secretion, B) the circadian clock of the adrenal gland is entrained by the autonomic nervous system (ANS) and C) the circadian clock of the adrenal gland and the autonomic ANS gates the sensitivity of the adrenal gland for ACTH. GC: glucocorticoids, ANS: autonomic nervous system [reproduced from Dickmeis (2009)].

4.1.9.4 The effects of corticosteroid hormones

Glucocorticoids have many therapeutic applications of which the most common is their application in the treatment of inflammatory diseases. Although natural and synthetic glucocorticoids may alleviate the symptoms associated with these diseases, long-term exposure to these steroids has many adverse effects. Figure 4.15 shows the major sites of glucocorticoid action in the human body.

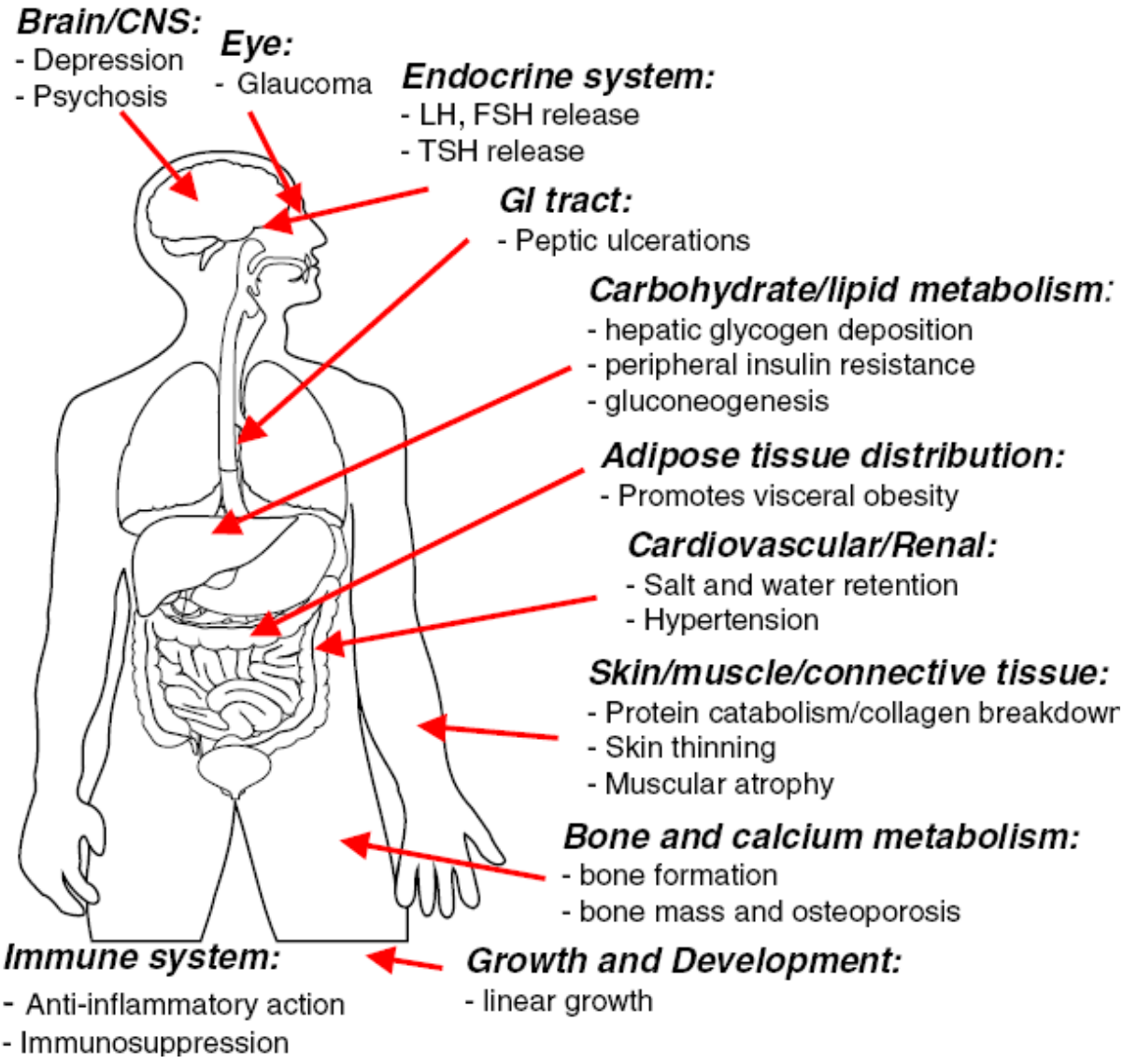


Figure 4.15: Major sites of glucocorticoid actions in the human body [reproduced from Arlt and Stewart, 2005].

4.1.9.4.1 Central nervous system

Glucocorticoids are able to effect behavior through interfering with normal brain metabolism. Clinical observations reveal that glucocorticoid deficiency and excess can lead to psychological disturbances such as euphoria, psycosis, depression, lethargy and apathy. GRs are ubiquitously expressed and the hydrophobic characteristics of glucocorticoids ensure that they are able to cross the blood-brain barrier. Although these steroids are able to cross this barrier, the P-glycoprotein pump has the ability to pump

several steroids, including cortisol, back across the blood-brain barrier [Ueda *et al* (1992); Karssen *et al* (2001)].

Studies have shown that large doses of glucocorticoids can lead to cell damage in the brain, specifically the hippocampus [Reagan & McEwen (1997)], due to calcium build up in the cells. Therefore, sustained elevated levels of glucocorticoids could induce various neurodegenerative diseases, such as Alzheimer's, by causing apoptosis of neurons. Steroid hormone administration and stressful experiences have been known to cause deficits in spatial and episodic memory and potentially play an important role in the modulation of long-term potentiation which is associated with memory formation [Leverenz *et al* (1999); Gould *et al* (1990)]. The latter explains the interest in glucocorticoids for the treatment of cognitive diseases [Salpolsky *et al* (1985)].

Studies have shown that the neuronal death caused by glucocorticoids may contribute to the decreased cognitive function associated with neurodegenerative diseases such as Alzeihemer's disease. DHEA, which is metabolized by CYP7B to its 7 α-hydroxylated metabolite, has known neuroprotective properties and is highly expressed in the brain [MacLusky *et al* (2004); Hajszan *et al* (2004)]. Individuals suffering from Alzeihemer's disease exhibit a significantly decreased CYP7B activity [Yau *et al* (2003)]. In the case of adrenal Cushing's syndrome, the production of DHEA is suppressed due to the damage caused by the glucocorticoids to the hippocampus. Although there have been suggestions that the loss of the protective properties of DHEA that is associated with Cushing's syndrome may result in a pronounced neurodegenerative effect, evidence supporting this is lacking.

In addition, glaucoma can be induced in genetically predipositioned individuals through glucocorticoid action. Glucocorticoids increase intraocular pressure by inhibiting fluid drainage from the eye. The underlying mechanism of this steroid-induced glaucoma is unknown [Clark (1995)].

The potential of glucocorticoids to treat inflammation and edema associated with CNS injury has also been explored. Studies have shown that glucocorticoid therapies have been effective treatments for ameliorating edema that are associated with brain tumors and to reduce the effects of bacterial meningitis, including loss of hearing, in human

subjects. However, the application of glucocorticoid therapies to intracerebral hemorrhage, traumatic brain injury, aneurysmal subarachnoid hemorrhage and ischemic stroke in clinical trials has not yet shown unambiguous therapeutic effects. Although glucocorticoid treatments have also been applied to spinal cord injury, the evidence supporting the use of glucocorticoid therapies is controversial [Gomes *et al* (2005)].

4.1.9.4.2 Cardiovascular

Mohinder *et al* showed that the administration of glucocorticoids caused a significant increase in cardiac output in normal and hypotensive subjects [Sambhi *et al* (1965)]. Although the pressor effects of deoxycorticosterone acetate and the consequential reduction of cardiac output were well documented at this time, the acute hemodynamic effects of high concentrations of glucocorticoids were not yet reported [Raab (1942)]. It was found that the administration of cortisone and ACTH for a period exceeding two weeks led to increased arterial pressure and no change in cardiac output. The interpretation of the results found in this study was complicated due to fluid retention and increased ventricular pressure caused by the increase in arterial pressure [Albert *et al* (1955)]. If cortisone is administered orally and only for a period of five days, the arterial pressure remains unchanged, cardiac output increases and peripheral resistance decreases [Hoffman & Emmrich (1959)]. Several studies have shown that the increase in cardiac output and the decrease in peripheral resistance resulting from glucocorticoid administration can be attributed to myocardial and peripheral effects [Sayers & Solomon (1960); Tanz & Kerby (1961)].

Although steroid-induced hypertension is suggested to be caused by water and salt retention through the action of mineralocorticoid receptors, this is not the case for the glucocorticoids [Panarelli *et al* (1998)]. It has been suggested that cortisol produces hypertension through sodium retention, although evidence to support this is lacking [Whitworth & Kelly (1995)]. Studies have shown that ACTH-induced hypertension in the rat is not affected by the presence of spironolactone at a concentration that inhibits water and sodium retention. In addition, ACTH-induced hypertension is also not affected by RU486 at a concentration that inhibits the metabolic effects of glucocorticoids [Li *et al* (1999]. This implies that glucocorticoid-induced hypertension is not a result of classic

glucocorticoid actions. Although the affects of cortisol on hypertension in humans is not clear, current investigations focus on the vascular effects produced by cortisol and the influence of this glucocorticoid on NO production [Kelly *et al* (1988); Whitworth *et al* (2000)].

High levels of glucocorticoids have also been associated with myocardial infarction. It has been suggested that glucocorticoids influence the signaling pathways of the β-adrenergic receptors, the uptake and metabolism of the catecholamines and the signaling pathways associated with IP_3, thus mediating myocardial infarction through these mechanisms [Adameova *et al* (2009)].

4.1.9.4.3 Metabolism

Glucocorticoids are well known for their ability to increase blood sugar levels by acting on protein, lipid and carbohydrate metabolism. The metabolic effects of glucocorticoids are depicted in Figure 4.16. Cortisol influences carbohydrate metabolism by increasing glycogen deposition through the inhibition of glycogen phosphorylase and the stimulation of glycogen synthase. Glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, the key enzymes in gluconeogenesis, are stimulated and hepatic glucose production is increased [Exton (1979)].

Cortisol activates lipolysis and the release of free fatty acids (FFAs) into the blood with a resulting increase in cholesterol and triglycerides, but a decrease in HDL. Lipoprotein lipase, leptin and glycerol-3-phosphate dehydrogenase are enzymes involved in adipogenesis that are stimulated by glucocorticoids. Chronically elevated glucocorticoid levels cause visceral obesity by stimulating adipose deposition and the latter is a symptom by which Cushing's syndrome can be identified [Bronnegard *et al* (1990)].

Elevated FFA levels inhibit glucose metabolism in muscle tissue by interfering with the normal action of glucagon and catecholamines, inducing hyperinsulemia [Björntorp (1991)]. In addition, insulin resistance can develop in muscle cells due to the inhibition of glycogen synthase and reduced concentrations of the fatty acid oxidation enzymes, citric acid synthase and β-hydroxyacyl-CoA [Patschan *et al* (2001)].

Cortisol disturbs the action of insulin in several different ways [Amatruda *et al* (1985)]. Studies have shown that cortisol inhibits the secretion of insulin from the β-cells of the

pancreas [Lambillotte *et al* (1997)], In addition it was shown that, in adipocytes, insulin resistance is induced through the modulation of insulin responsive glucose transport after synthetic glucocorticoids were administered [Garvey *et al* (1989)]. Synthetic glucocorticoids, such as dexamethasone, inhibit the translocation of the glucose transporters to the cell membranes mimicking the symptoms associated with insulin resistance [Rosmond (2003)]. The metabolic effects of glucocorticoids are depicted in Figure 4.16.

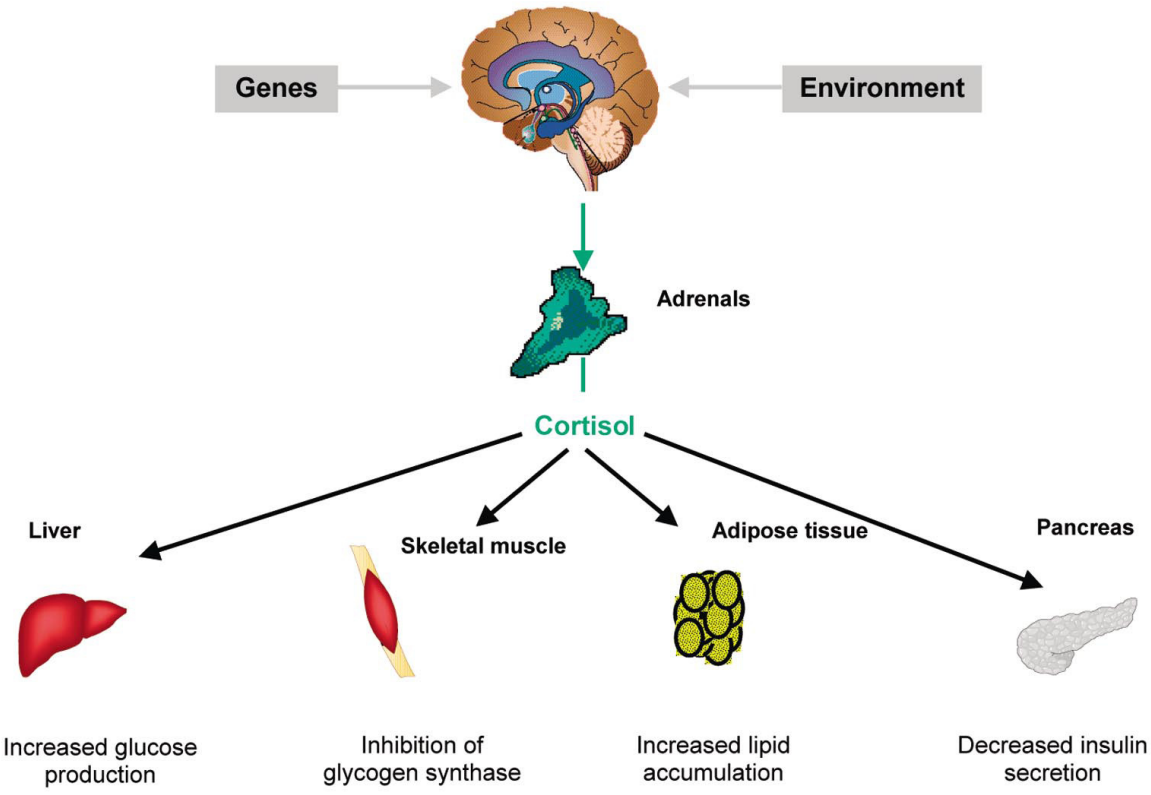


Figure 4.16: Activation of the HPA axis by environmental and genetic factors in prone individuals, results in visceral obesity and insulin resistance [Rosmond (2003)].

4.1.9.4.4 Immune system

The immune response is inhibited by the action of glucocorticoids which reduce lymphocyte numbers in the blood by stimulating the redistribution of these lymphocytes into various tissues, such as the spleen, bone marrow and lymph nodes, while increasing

neutrophil numbers [Glaser & Kiecolt-Glaser (2005)]. Glucocorticoids stimulate lymphocyte apoptosis and inhibit immunoglobulin synthesis, thereby directly influencing T and B lymphocytes which are responsible for the production of cytokines. By binding to NF-κB directly, glucocorticoids inhibit the action of NF-κB and cause the inhibition of cytokine production from lymphocytes [McKay & Cidlowski (1999)]. Based on the inhibitory actions of glucocorticoids on the immune system, several potent synthetic glucocorticoids have been designed to treat autoimmune diseases.

Other anti-inflammatory effects mediated by glucocorticoids include cytotoxic activity, inhibition of monocyte differentiation, prevention of histamine and plasminogen activators and the inhibition of prostaglandin synthesis [Peers & Flowers (1990)].

4.1.9.4.5 Skeletal and muscular growth and development

Although glucocorticoids induce gene transcription of GH, excess glucocorticoids stimulate the breakdown of muscle, connective tissue and bone. Thus, the inhibitory effect of glucocorticoids is due to increased concentrations of GRs in muscle, connective and bone tissue and the inhibition of glycogen synthase [Dallman *et al* (1993); Holmäng & Björntorp (1992)].

Glucocorticoids inhibit Ca^{2+} absorption in the gastrointestinal tract and increases renal Ca^{2+} excretion. The increased renal excretion is suggested to be caused by the inhibition of tubular re-absorption. Although there have been consistent results regarding the investigation of the glucocorticoid-induced increased renal Ca^{2+} secretion, this is not the case for intestinal Ca^{2+} re-absorption. The observed inconsistent results are attributed to the differential effects of steroids in the bowel [Lukert & Raisz (1990)].

The consequential negative Ca^{2+} balance in the body causes hypersecretion of the parathyroid hormone (PTH) [Klein *et al* (1977); Paz-Pacheco *et al* (1995)] which is a significant factor contributing towards the pathogenesis of osteoporosis. Glucocorticoids stimulate PTH secretion in a dose and time dependant manner and alter PTH sensitivity within the osteoblasts and osteoclasts. Consequently, a smaller concentration of PTH is necessary to elicit biochemical changes [Wong (1979)]. Although glucocorticoid treatment has the potential to increase PTH sensitivity in osteoclasts and osteoblasts, no increase in PTH concentrations has been reported in individuals treated with

glucocorticoids. Therefore, the induction of osteoporosis by glucocorticoid therapy cannot be attributed to the hyperparathyroidism only [Hattersley *et al* (1994); Luengo *et al* (1991); Paz-Pacheco *et al* (1995)].

Bone loss resulting from long-term glucocorticoid therapy is not uncommon. The proposed mechanisms of glucocorticoid-induced osteoporosis are shown in Figure 4.17. Long-term exposure to glucocorticoid therapy alters the proliferative and metabolic activities of osteoblasts and osteoclasts resulting in decreased bone formation. Subjects treated with dexamethasone exhibited decreased concentration of serum osteocalcin and type 1 procollagen [Reid (1997)]. The inhibition of the metabolism and proliferation of osteoblasts have been documented in several species [Delany *et al* (1996); Ziegler & Kasperk (1998); Subramaniam *et al* (1992)].

Glucocorticoids inhibit the synthesis of inflammatory mediators, specifically cytokines, which are involved in the metabolism of bone matrix proteins. Interleukin (IL)-1 and tumor necrosis factor (TNF) α and β are potent stimulators of osteoclastogenesis, therefore these proinflammatory cytokines increase bone resorption and decrease bone formation [Teitelbaum (2000)]. Considering that the synthesis of T-lymphocytes and monocytes, which are responsible for the production of IL-1 and TNF, is inhibited by glucocorticoids, the bone loss resulting from glucocorticoid therapy cannot be attributed to the effects of IL-1 and TNF. Through eliciting an anti-inflammatory effect, glucocorticoids inhibit inflammatory mediators resulting in reduced bone loss [Russell (1993)]. This mechanism is thus not responsible for glucocorticoid induced osteoporosis.

Several studies have indicated that glucocorticoids reduce mRNA levels of bone matrix proteins and insulin-like growth factor (IGF)-1. IGF-1 is significant since it modulates metabolic activities in osteoblasts [Hofbauer *et al* (1999)] thus mediating osteoblast functions, osteoblast-osteoclast interactions and osteoclastic functions. Glucocorticoids are known to inhibit IGF and IGF binding protein synthesis by reducing the expression of IGF and the IGF binding proteins [Chevalley *et al* (1996)]. In addition, glucocorticoids have been documented to reduce osteoblastogenesis, thus adding to the loss of bone density caused by long-term exposure to glucocorticoids [Lian *et al* (1997)].

The data regarding the effects of glucocorticoids on osteoclasts has been inconsistent and inconclusive. Overall, glucocorticoid-induced osteoporosis develops due to a loss of bone integrity and decreased bone formation. The mechanisms in which excess glucocorticoids effect the development of osteoporosis is shown in Figure 4.17.

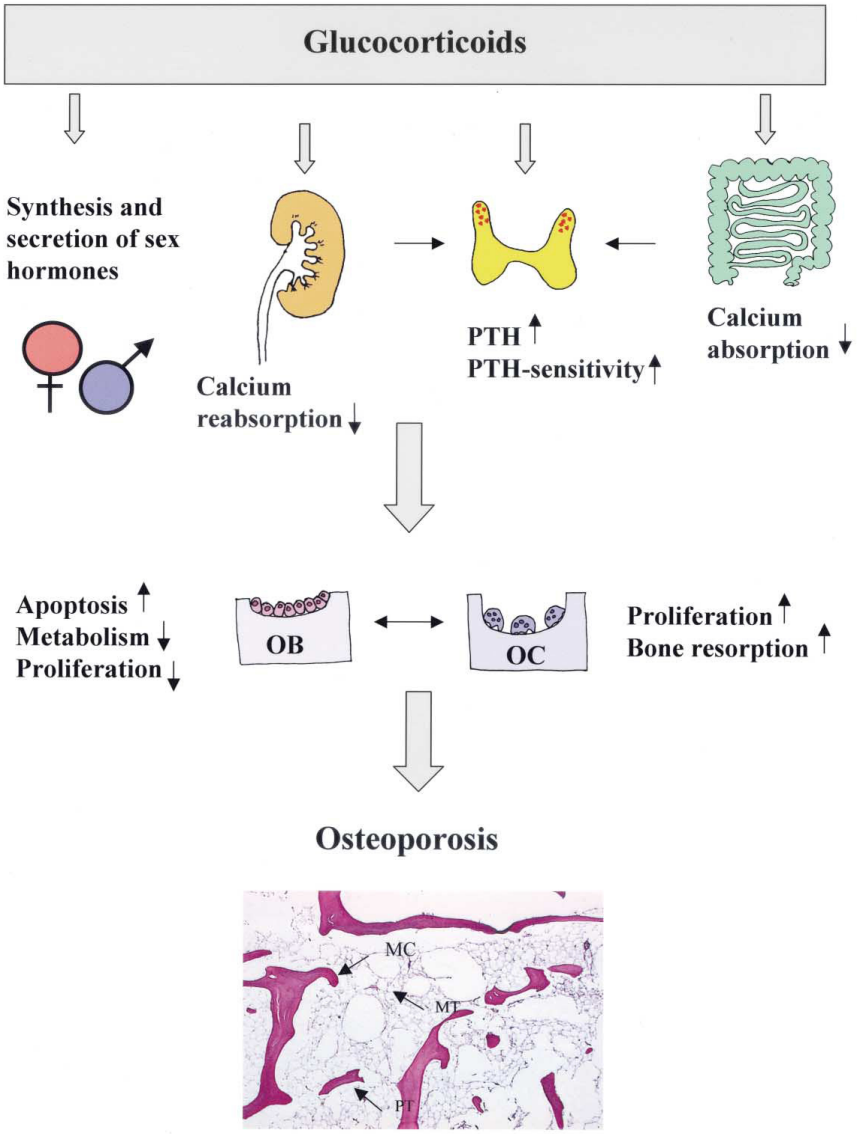


Figure 4.17: Mechanisms of glucocorticoid-induced osteoporosis. MC: microcallus, OB: osteoblasts, OC: osteoclasts, PTH: perforated trabecular structure, MT: missing trabecular structure [reproduced from Patschan *et al* (2001)].

In muscle tissues, glucocorticoids cause insulin resistance and atrophy [Leibovich & Ross (1975)]. Studies have shown that exposure to glucocorticoids affect the numbers of both

type I and type IIb muscle fibers, although acute atrophy of only type IIb fibers was observed. Also, the exposure to glucocorticoids causes significant changes in the metabolism of muscle cells. Although the concentrations of lactate dehydrogenase appear normal, the glycogen synthase concentrations were low compared to healthy muscle cells implying the development of insulin resistance. Fatty acid oxidation is affected through lowered concentration of citric acid synthase and β-hydroxyacyl-CoA in muscles obtained from patients receiving glucocorticoids [Danneskiold-Samsoe & Grimby (1986)]. The metabolic changes associated with glucocorticoid excess result in considerable loss of muscle strength which is linked to glucocorticoid-induced osteoporosis. The muscle weakness causes the loss of the normal forces exerted by the contraction of the muscles that support the bone structure and thereby contribute to bone loss [Luckert and Raisz (1990); Patschan *et al* (2001)].

4.1.9.4.6 Other

Glucocorticoids suppress the actions of the thyroid hormones and consequently affect the secretion of thyroid-stimulating hormone (TSH). Corticosteroids inhibit the conversion of thyroxine to triiodothryronine, the active hormone, by interacting with the 5'deiodinase. Glucocorticoids also inhibit the secretion of follicle stimulating hormone (FSH), gonadotrophin-releasing hormone (GnRH) and luteinizing hormone (LH) [Chrousos (1998)] which may cause reproductive problems.

Glucocorticoids affect the actions of the kidney in various ways which consequently leads to elevated blood pressure [Fraser *et al* (1989)]. Glucocorticoids increase the pressure sensitivity of vascular smooth muscle by making it more susceptible to agents like epinephrine, norepinephrine and angiotensin II and by decreasing endothelial dilation.

In the kidneys, cortisol promotes potassium loss and sodium retention in the distal nephron while it also increases glomerular filtration, free water clearance and epithelial sodium transport [Marver (1984)]. Hyponatremia is caused by glucocorticoids that act as antagonists to vasopressin and is seen in glucocorticoid deficient individuals.

Chronically elevated glucocorticoid levels can cause peptic ulcer disease and pancreatitis by interfering with epithelial ion transport. As mentioned previously, Ca^{2+} absorption is

decreased and renal Ca^{2+} excretion is increased by the presence of excess glucocorticoids [Presley & Funder (1975)]. It has been shown that dexamethasone induces the uptake of water and sodium ions, but significantly decreases the uptake of potassium in the rat and human colonic epithelial [Sandle & McGlone (1987)].

4.2 Conclusion

Stress induces a cascade of different reactions in the body which causes increased glucocorticoid secretion from the adrenal cortex and consequently an increase in free circulating cortisol. Glucocorticoids, cortisol and corticosterone, are essential hormones without which life will not be possible and their main function is to regulate blood glucose levels. Cortisol and corticosterone also play a vital role in the stress response by regulating the response through the negative feedback inhibition of the HPA axis. Long-term exposure to glucocorticoids causes transcriptional modifications whereas acute exposure to glucocorticoids affects hormone secretion. The regulation of the stress response through negative feedback inhibition of the HPA axis by glucocorticoids can be overcome when an organism is in a state of chronic stress. The HPA axis becomes desensitized to inhibition by cortisol, and cortisol production and secretion is sustained. This causes long-term elevated plasma cortisol levels which may cause many adverse effects including metabolic disorders, depression, anxiety, cardiovascular ailments, glaucoma, immune-suppression and osteoporosis. The implication of a dysfunctional HPA axis in the adverse effects associated with long-term glucocorticoid exposure could provide a target for the treatment and improvement of therapies for these effects. In addition, a better understanding of the molecular and physiological mechanisms underlying the adverse disorders associated with long-term glucocorticoid exposure could lead to a breakthrough in the improvement of therapies for these disorders. Cortisol is produced by the P450 enzymes in the adrenal cortex. This implies that targeting the P450 enzymes may reduce the production of cortisol and alleviate the ailments associated with elevated cortisol levels.

P450 enzymes are highly specific towards their natural substrate and therefore, a potential inhibitor, be it a competitive or non competitive inhibitor, will potentially have a similar structure to the natural steroid substrate. A substance with a similar structure may have

the potential to bind to the hydrophobic heme pocket in the active site of the enzymes. Besides occupation of the enzyme's active site, P450 enzymes can also be inhibited or rendered inactive by influencing the electron transporters.

P450 enzymes have been inhibited by plant extracts suspected of containing compounds that structurally resemble the natural steroid substrates. Perold showed that extracts made from fermented and unfermented rooibos inhibited the catalytic activity of P450 enzymes, CYP17 and CYP21. The results obtained identified flavonoid compounds with structural similarity to the steroid substrates, progesterone and pregnenolone, and showed that the flavonoid compounds also influenced the activity of CYP17 and CYP21 [Perold, MSc thesis, 2009]. Prevoo *et al* showed that CYP17 and CYP21 were influenced by *S. frutescens* extracts and several other compounds within these plant extracts. It was suggested that the extracts could reduce cortisol levels by influencing these enzymes [Prevoo *et al* (2005)].

The following chapter investigates the effects of *S. frutescens* extracts, and fractions thereof, on the substrate binding and catalytic activity of CYP11B1, the enzyme responsible for the conversion of deoxycortisol and DOC to produce cortisol and corticosterone, respectively. By potentially inhibiting CYP11B1, the extracts of *S. frutescens* may have the potential to alleviate the symptoms associated with elevated cortisol levels. The identification of compounds within *S. frutescens* that share structural similarity with the natural steroid substrates may also provide insight into the mechanisms by which extracts from this plant affect cytochrome P450 enzymes.

Chapter 5

The influence of *S. frutescens* on cytochrome P450 11β-hydroxylase

5.1 Introduction

S. frutescens is a medicinal plant common to South Africa. Extractions made from the dried material of this plant are traditionally used to treat the symptoms of various diseases of which stress and diabetes are the most renowned. Although whole *S. frutescens* extracts are traditionally used to treat the symptoms of these ailments, certain compounds have been identified within these extracts that could warrant their use in treatment of stress, depression, diabetes, cancer and inflammation [Smith (1895); Watt & Breyer-Brandewijk (1962)]. These compounds include GABA (anti-depressant and anti-anxiety) [Ortega (2003)], canavanine (anti- cancer) [Seier *et al* (2002)], pinitol (anti-diabetic and anti-inflammatory) [Brümmerhoff (1969)] and various flavonoids, saponins and triterpenoids (mostly unknown effects) [Snyders (1965)]. Recently identified sutherlandiosides (cycloartanes) are presumably involved in the corticometric effects elicited by *S. frutescens* extracts [Fu *et al* (2008)].

During the normal stress response, the HPA axis is activated by a stressor which increases basal plasma glucocorticoid concentrations through a cascade of reactions. After the stress stimulus ceases, the HPA axis is inhibited through a negative feedback inhibition by glucocorticoids. However, during chronic stress, the glucocorticoid receptors in the brain are desensitized, resulting in an impaired negative feedback mechanism which ultimately leads to chronically elevated plasma glucocorticoid concentrations. The long-term exposure to elevated plasma glucocorticoid levels has many adverse effects which include amongst others, depression, diabetes, inflammation, osteoporosis and cardiac disease that may have severe consequences [Chrousos (1998)]. The regulation of plasma glucocorticoid levels is therefore vital.

The use of *S. frutescens* to treat symptoms of stress has been linked to its ability to reduce plasma glucocorticoid levels, presumably through interfering with the negative feedback inhibition of the HPA axis and/or by inhibiting the steroidogenic P450 enzymes, CYP17 and CYP21 [Prevoo *et al* (2004); Smith & Myburgh (2004)]. Smith and Myburgh

observed reduced basal plasma glucocorticoid levels in rats subjected to immobilization stress after administration of *S. frutescens* extracts. The extracts also induced an increase in plasma glucocorticoid concentrations in rats that were not subjected to immobilization stress. It is possible that glucocorticoid production may be modulated by *S. frutescens* extracts thus influencing the HPA axis. Prevoo *et al* subsequently demonstrated that *S. frutescens* extracts inhibit P450 enzymes, CYP17 and CYP21 that are responsible for the production of glucocorticoid precursors, possibly influencing normal glucocorticoid production.

The aim of this study was to investigate the influence of *S. frutescens* extracts on the activity of CYP11B1, which catalyses the final step in the biosynthesis of corticosterone and cortisol. The influence of a methanol extract of *S. frutescens* was investigated, as well as various fractionations of this extract in an attempt to identify compounds that may be responsible for the bioactivity of *S. frutescens.* The bioactivity of the *S. frutescens* extract and extract fractions was assayed using spectral binding assays to determine the inhibition of substrate binding to the enzyme. The binding of DOC and deoxycortisol to CYP11B1 was investigated in ovine adrenal mitochondria in the presence of the extracts and the extract fractions. Substrate conversion assays were subsequently conducted in COS1 cells to investigate the ability of *S. frutescens* extracts and fractions to inhibit the conversion of DOC and deoxycortisol to corticosterone and cortisol, respectively. Since inhibition of the catalytic activity of CYP11B1 influences the production of glucocorticoids directly it is possible that *S. frutescens* extracts and its fractions may influence plasma levels, indicating an application in the treatment of the symptoms of stress.

5.2 Material and methods

5.2.1 Reagents

DOC, deoxycortisol, Dulbecco's modified Eagle's medium (DMEM), Hepes, chloroquine and diethylaminoethyl-dextran were purchased from Sigma Chemical Co.(St Louis, MO, USA). COS1 cells were purchased from the American Type tissue Culture Collection (Manassas, VA, USA). Penicillin-streptomycin, trypsin-EDTA and

Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). Mirus transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Fetal calf serum was purchased from Highveld Biological (Lyndhurst, RSA) and lysogeny/Luria broth (LB) medium was purchased from Difco Laboratories (Detroit, MI, USA). Nucleobond AX plasmid preparation kits were purchased from Macherey-Nagel (Duren, Germany) and Pierce BSA protein assay kits were purchased from Pierce (Rockford, IL, USA). All chemicals used were of high analytic grade.

Sutherlandia samples were collected from the Western Cape and identified as *Sutherlandia frutescens* (Prof B-E van Wyk, Dept. Botany, University of Johannesburg, South Africa). SU1 was a kind gift from B-E van Wyk. flavonoid standard compounds vitexin, orientin and rutin were donated by Prof Wentzel Gelderblom from the MRC, Western Cape. Sheep adrenals were collected from Paarl Abattoir in Paarl, Western Cape, South Africa. The surgically removed adrenals were placed on ice immediately after removal and before use.

5.2.2 Preparation of *S. frutescens* extracts

Aqueous extracts of dried ground *S. frutescens* were prepared by boiling 2.16 g dried *S. frutescens* plant material in 275 ml de-ionized water for 30 minutes. The extract was filtered using Whatman No.4 filter paper followed by a 0.45 µm pore size membrane filter (MilliPore), and subsequently lyophilized overnight. The dried extract (158.6 mg) was resuspended in 13 ml de-ionized water to a final concentration of 122 mg/ml and stored in 2 ml aliquots at 4 ºC. Methanol extracts of dried ground *S. frutescens* were prepared by placing 18.6 g dried *S. frutescens* plant material in a glass soxhlet extractor. The extractor was fitted with a double walled condenser and placed on a round bottom flask containing 250 ml chloroform ($CHCl_3$). The plant material was subsequently extracted for 8 hours on a heating mantle. After 8 hours, the chloroform phase was discarded and replaced with 250 ml methanol. After the plant material was extracted for an additional 8 hours, the methanol fraction was removed and dried on the rotary evaporator under reduced pressure. The dried extract (1603 mg) was resuspended in 30

ml de-ionized water and clarified by centrifugation at 6000 x g for 5 minutes. The supernatant was stored in 5 ml aliquots at 4 ºC. The final concentration was 53.43 mg/ml. In an attempt to identify compounds to which the bioactivity *S. frutescens* may be attributed, two strategies were considered: firstly, the sequential extraction of the methanol extracts of *S. frutescens* with petroleum ether, ethyl acetate and butanol and, secondly, the fractionation of the methanol extract of *S. frutescens* by HPLC.

5.2.3 Solvent extraction of a methanol extract obtained from *S. frutescens*

An extract of *S. frutescens* was prepared by extracting 18.6 g dried plant material with methanol using a glass soxhlet extractor and re-dissolving the vacuum dried residue in de-ionized water as described above (section 5.2.2). The methanol extract of *S. frutescens* (5 ml) was subsequently subjected to sequential extractions with equal volumes (5 ml of each) of petroleum ether, ethyl acetate and butanol using a separation funnel. Each extraction was performed by shaking the funnel vigorously for 1 minute after which the phases were allowed to separate. Petroleum ether and ethyl acetate extracts were dried on a rotary evaporator and the butanol and residual aqueous extracts were lyophilized over night. The dried extracts were resuspended in 1 ml ethylene glycol (Table 5.1) and filtered to remove any undissolved residue.

5.2.4 HPLC fractionation of the methanol extract of *S. frutescens*

An extract of *S. frutescens* was prepared by extracting 18.6 g dried plant material with methanol using a glass soxhlet extractor as described above (section 5.2.2) and subjected to HPLC fractionation using a Waters (Milford, MA, USA) high performance liquid chromatograph coupled to a Waters 991 Photodiode Array detector. The extract, 100 µl, was chromatographed using a Novapak® C18 reverse phase column (1.7 µm, 2.1 mm x 50 mm). The solvent systems used to elute the fractions were: deionised water (solvent A) and methanol (solvent B). A linear gradient was run from 100 % A to 100 % B in 40 minutes at a flow rate of 2.5 ml/minute. The eluate was monitored at 420 nm and five fractions were subsequently collected. Two millilitres of the methanol extract of *S. frutescens* was fractionated and the collected fractions were lyophilized and resuspended in 2 ml de-ionized water. The resuspended fractions were filtered. The final

concentrations of fractions 1-5 are shown in Table 5.2. The fractions were subsequently analysed by LC-MS.

5.2.5 Preparation of adrenal mitochondria

Mitochondria were isolated from ovine adrenals according to the method by Yang & Cederbaum (1997) by differential centrifugation [Yang & Cederbaum (1997)]. Procedures for the mitochondrial isolation from the ovine adrenals were conducted at 4 ºC.

The adrenals from freshly slaughtered sheep were decapsulated and washed with a 15 mM KCl solution. The adrenal tissue (51.36 g) was homogenized in 100 ml 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA in a Hamilton Beech blender followed by a Potter-Elvejhem glass homogenizer. The homogenate was centrifuged for 15 minutes at 12 000 x g at 4 ºC to sediment the mitochondria. The mitochondrial pellet was resuspended in 200 ml 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 0.25 M sucrose and 1 % BSA, and subsequently homogenized again. The homogenate was centrifuged for 15 minutes at 12 000 x g at 4 ºC. To wash the mitochondrial pellet, the pellet was resuspended in 200 ml 10 mM Tris-HCl buffer (pH7.4) containing 1 mM EDTA, 0.25 M sucrose and 1 % BSA and homogenized. The washing step was repeated twice. After centrifugation, the washed mitochondrial pellet was lyophilized overnight and the mitochondrial powder was stored at -18 ºC. The final dry mass of the mitochondrial powder was 0.95 g.

5.2.6 Determination of the mitochondrial cytochrome P450 concentration

The method used to determine the cytochrome P450 concentration of the mitochondrial powder was previously described by Omura & Sato [Omura & Sato (1964)]. The mitochondrial powder (2 mg/ml) was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 10 % ethylene glycol and sonicated, on ice, for 5 minutes with 1 minute intervals. The mitochondrial sonicate was saturated with carbon monoxide and 1 ml of sonicate was added to each of two optically matched cuvettes (sample and the reference). A baseline was recorded between 400 and 500 nm. Sodium dithionate (~2 µg) was subsequently added to the sample cuvette and an initial spectrum was recorded between

400 and 500 nm. After 10 minutes the spectrum had developed completely and a final recording was made.

The cytochrome P450 concentration of the mitochondrial solution was determined by using a molar extinction coefficient (ε) = 91 cm^{-1} mM^{-1} in the following equation:

$$\Delta \text{ Abs} = \varepsilon c l \qquad \text{(Eq. 1)}$$

where ΔAbs represents the change in absorbance between 450 and 500 nm, *l* represents the path length through the solution (1cm) and c represents the concentration of the cytochrome P450 enzymes [Yang & Cederbaum (1997)]. A Pierce BSA protein assay kit (Perstorp Life Sciences Company, IL, USA) was used to determine the protein concentration of the mitochondrial suspension, using a Titertek Multiscan PLUS spectrophotometer to determine the absorbance at 540 nm. The final protein concentration of the mitochondrial suspension was 0.76 mg protein/mg mitochondrial powder.

5.2.7 Spectral binding assays

Spectral assays were performed to determine whether the binding of steroid substrates to the ovine adrenal mitochondrial P450 enzyme, CYP11B1, was influenced by the presence of *S. frutescens*.

5.2.7.1 Substrate-induced difference spectra

These experiments were performed at room temperature using a Cary 100 Conc UV-Visible Spectrophotometer by Varian in a final reaction volume of 1 ml. The cytochrome P450 concentration of the ovine adrenal mitochondrial suspension was 0.88 µM. One ml of the mitochondrial suspension was pipetted into two optically matched cuvettes and a baseline was recorded between 370 and 450 nm. A tandem configuration for the sample and reference cuvettes was required to rule out the absorbance of the test components in the Soret region. The steroid substrates, DOC and deoxycortisol, were dissolved in ethanol and 2 µl of substrate solution was added to the sample cuvette. An equal amount (2 µl) of ethanol was added to the reference cuvettes, the cuvettes were gently inverted and a spectrum recorded [Prevoo *et al* (2005)]. Inhibition of substrate binding to

CYP11B1 was studied by adding equal volumes of the plant extract to the reference and sample cuvettes prior to the recording of the baseline.

The potential inhibition of substrate binding was determined by preparing mitochondrial suspensions containing different concentrations of the methanol extract of *S. frutescens* (0.21 mg/ml – 1.68 mg/ml) in the presence of 8 µM and 32 µM DOC.

Once the inhibitory effect of the methanol extract of *S. frutescens* was determined, the HPLC fractions were investigated. The fractions were added to the mitochondrial solution at concentrations comparable to fractions in the original methanol *S. frutescens* extract (volume/volume). A substrate concentration of 8 µM was added to the mitochondrial suspension.

Four sutherlandiosides (SU 1-4) have been identified in extracts of *S. frutescens* and it has been suggested that these compounds contribute to the corticometric effects of *S. frutescens*. To investigate whether these corticometric effects are elicited through interfering with substrate binding to CYP11B1 a spectral binding assay was performed in the presence of SU1. SU1 was dissolved in 1 % ethanol and added to the cuvette to a final concentration of 2.4 µM. The percentage inhibition was calculated by using the following Equation:

$$\% \text{ inhibition} = \{(A - B)/ A\} \times 100 \qquad \text{(Eq.2)}$$

where A represents the difference in amplitude between the absorbance at 390 nm and 420 nm of the spectra containing no test compound and B represents the difference in amplitude between the absorbance at 390 nm and 420 nm of the sample containing the test compound [Wilson & Walker (2000)].

5.2.8 Substrate conversion assay in COS1 cells

The influence of *S. frutescens* on the catalytic activity of baboon CYP11B1 was investigated by expressing the recombinant enzyme in COS1 cells: a non-steroidogenic, tumor cell line derived from the African green monkey kidney.

5.2.8.1 DNA isolation

The DNA constructs were isolated from *E. coli* transformed with the following vector constructs: baboon CYP11B1/pTarget, human ADX/pTarget, angora CYP17/pVector and pCI-neo.

An overnight culture was prepared containing 100 ml of LB medium, 100 µl of transformed *E. coli* strain and 100 µg/ml ampicillin. The culture was allowed to grow for 16 hours, where after it was centrifuged at 5930 x g for 15 minutes at 4 ºC to remove the remaining LB medium. The DNA was isolated from the bacterial pellet using a Nucleobond AX plasmid preparation kit, according to the manufacturer's instructions. The final concentration of the isolated recombinant DNA is shown in Table 5.4.

5.2.8.2 Maintenance of COS1 cells

COS1 cells were incubated at 37 ºC, 5 % CO_2 and 90 % humidity in DMEM (high glucose containing 15 % $NaHCO_3$, 10 % fetal calf serum and 1 % penicillin streptomycin solution). The addition and removal of media was performed in a sterile laminar flow hood.

A 1 ml frozen cell culture was thawed at room temperature and resuspended in 10 ml of culture medium, pre-heated to 37 ºC. The cell suspension was plated into a 100 mm culture dish and incubated. The culture media was replaced daily with 10 ml of warmed culture medium until the cells were confluent (approximately 4 days).

Confluent cells were split as follows into 100 mm culture dishes: The medium was removed from the culture dish and the cells were washed with 1 ml warmed trypsin-EDTA medium containing 10 % Dulbeco's PBS, without calcium and magnesium, and 10 % trypsin-EDTA. Trypsin-EDTA, 1 ml was pipetted into the dish and the cells were incubated for 3 minutes before the cells were harvested. The harvested cells were resuspended in 30 ml of culture medium and plated into three 100 mm culture dishes until the cells were confluent.

Freezer stocks were prepared after at least 3 cycles of confluence as follows: The cells were collected from the 100 mm culture dishes as described above and centrifuged at 500 x g for 5 minutes. The media was removed and the cells were resuspended in 3 ml freeze

medium (culture medium containing 10 % DMSO) for every 100 mm dish. The 1 ml aliquots were stored at -80 ºC for 48 hours, where after the aliquots were stored in liquid nitrogen until further use.

5.2.8.3 Transfection of COS1 cells

Confluent cells were also seeded into 12 well (2.5 cm) culture plates after the second cycle of confluence, 1 day prior to transfection, at a density $1x10^5$ cells per ml. On the day of transfection, the culture media was removed and replaced with fresh media. Culture medium without fetal calf serum (50 µl per well) was pipetted into a sterile microcentrifuge tube and incubated for 10 minutes with Mirus transfection reagent (1.5 µl per well). Plasmid DNA, 0.5 µg per well, was added to the reaction mixture and incubated for 20 minutes, where after 50 µl of reaction mixture was added to each well. All the incubations in the transfection procedure were performed at room temperature. The cells were co-transfected with the CYP11B1 and ADX plasmid construct to determine the influence of *S. frutescens* on the conversion of DOC and deoxycortisol. In the control experiments the cells were (i) co-transfected with the angora CYP17 and pCIneo plasmid construct to assess transfection efficacy, and (ii) pCIneo to ensure that the cells did not metabolize the steroid substrate in the absence of additional DNA constructs. The pCIneo vector contained no insert DNA. The transfected cells were incubated for 72 hours before substrate addition.

5.2.8.4 Substrate conversion assay

The culture medium was replaced with culture medium containing 1 µM substrate which initiated the conversion reaction. The substrate was also added to cells which had not been transfected to monitor any activity towards the substrate by endogenous enzymes.

Several dilutions of the methanol *S. frutescens* extract were added to the steroid medium to assess the effect of this extract on the catalytic activity of baboon CYP11B1. The final concentration of the methanol *S. frutescens* extracts were 2.4 mg/ml, 4.8 mg/ml and 12.2 mg/ml.

The dried extracts of the petroleum ether, ethyl acetate, butanol and the residual aqueous extract were resuspended in ethylene glycol and added to the steroid medium in such a

way that their volumes would be comparable to that of the methanol extract of *S. frutescens*. For every 5 ml methanol extract extracted with petroleum ether, ethyl acetate and butanol, the dried extract was resuspended in 1 ml ethylene glycol. Therefore 10 µl of each of the petroleum ether, ethyl acetate, butanol and residual aqueous extracts were added for every 50 µl (10.68 mg/ml) of methanol extract of *S. frutescens*. These volumes were equivalent to 0.58 mg/ml petroleum ether, 0.52 mg/ml ethyl acetate, 0.25 mg/ml butanol, 0.19 mg/ml residual and 2.66 mg/ml methanol extract of *S. frutescens.*

The methanol fractions (Fractions 1-5) were added to the steroid medium to assess the effect on the catalytic activity of baboon CYP11B1 in a volume that would be comparable to the original methanol extract of *S. frutescens*.

Flavonoid compounds that have previously been identified in medicinal plants were also assayed. Rutin, vitexin and orientin were dissolved in ethylene glycol and added to the substrate medium to a final concentration of 10 µM each.

5.2.8.5 Extractions of steroid substrate and metabolites

Media aliquots, 50 µl, were removed from each well of the culture plate at specific time intervals, added to 5 ml dichloromethane and vortexed. The samples were centrifuged, followed by the aspiration of the aqueous phase and drying of the dichloromethane phase under nitrogen gas. The dried steroids were resuspended in 120 µl methanol and analyzed by LC-MS.

On completion of the assay, the culture medium was removed and the culture dishes were washed with PBS to remove residual medium. The cells were collected and lysed by sonication and the protein concentration was subsequently determined using a Pierce BSA protein assay kit.

5.2.8.6 Cell viability assay

The viability of the COS1 cells in the presence and absence of the *S. frutescens* extracts was determined by investigating the effect of these extracts on the ability of the COS1 cells to convert testosterone to androstenedione through endogenous 17β-hydroxysteroid dehydrogenase activity [Trant (1994)]. Substrate medium containing 1 µM of testosterone was added to confluent COS1 cells in the presence and absence of *S.*

frutescens (12.2 mg/ml). After a 24 hour period, 50 µl steroid medium was removed, extracted and redissolved in 120 µl methanol for analysis by LC-MS.

5.2.8.7 LC-MS

An ACQUITY UPLC (Waters, Milford, USA) was used in combination with an API Quattro Micro tandem mass spectrometer (Waters, Milford, USA) for the atmospheric pressure-chemical ionization interface in positive mode to analyze the presence of steroids. The total run time per sample was 5 minutes at a flow rate of 0.4 ml/minute and the injection volumes of standards and samples was 5 µl. The corona pin was set to 7 µA, the cone voltage 30 V and APCI probe temperature was 450 ˚C. All other settings were optimized to obtain the strongest signal possible. Calibration curves were constructed by using weighted (1/*x*2) linear least squares regression. Data was collected with the MassLynx 4.0 software program.

5.2.9 Statistical analysis

The results were analyzed with GraphPad prism Version 5.0 (GraphPad Software, San Diego, California, USA). The results shown are the mean of three independent experiments and the error bars represent the standard errors of the means (S.E.M).

5.3 Results

The bioactivity of *S. frutescens* was assayed using spectral binding and conversion assays. An aqueous infusion and a methanol extract were prepared and bioactivity assays were subsequently carried out using the methanol extract of *S. frutescens*. The methanol extract was therefore assayed, where after the extract was subjected to sequential extraction with petroleum ether, ethyl acetate and butanol. These extracts, as well as the fractions obtained by HPLC fractionation of the methanol extract, flavonoid compounds and a cycloartane glycoside, sutherlandioside A (SU1), were assayed for bioactivity. The spectral binding assays assessed the effect *S. frutescens* has on the ability of steroid substrates, DOC and deoxycortisol, to bind to adrenal mitochondrial CYP11B1. The binding of a natural steroid substrate to a P450 enzyme induces a change in the overall spin equilibrium of the enzyme. The change in spin state results in a type I difference

spectrum with a peak maximum at 390nm and a peak minimum at 420nm, which is detectible by UV spectrometry. A reduction in the amplitude of the type I difference spectrum indicates the inhibition of enzyme binding to its substrate.

The ability of *S. frutescens* to interfere with the catalytic activity of baboon CYP11B1 was subsequently investigated in transiently transfected COS1 cells. The percentage conversion of DOC and deoxycortisol to corticosterone and cortisol, respectively, in the absence and presence of *S. frutescens* extracts was used to assess the bioactivity of *S. frutescens*. The petroleum ether, ethyl acetate, butanol extractions as well as the residual aqueous extract, fractionations of the methanol extract and flavonoid compounds were also assessed for bioactivity by determining their potential inhibitory effect on the ability of CYP11B1 to convert DOC to corticosterone.

Prevoo *et al* showed the methanol extract of *S. frutescens* to be the more potent inhibitor of CYP17 and CYP21 compared to the aqueous extract [Prevoo *et al* (2004)]. It cannot, however, be assumed that the methanol extract would also be the stronger inhibitor of CYP11B1.

5.3.1 LC-MS analysis of *S. frutescens*

S. frutescens is consumed as an aqueous extraction and to determine whether the aqueous extract and the methanol extract of *S. frutescens* differed significantly according to their hydrophilic and hydrophobic compound content, these extracts were analyzed by LC-MS (Figure 5.1). Although there was little difference between the chromatograms of these extracts, the methanol extract was used for downstream experiments since the LC-MS profile of this extract indicated the presence of hydrophilic compounds that are not present in the aqueous extract (indicated by the brackets in Figure 5.1).

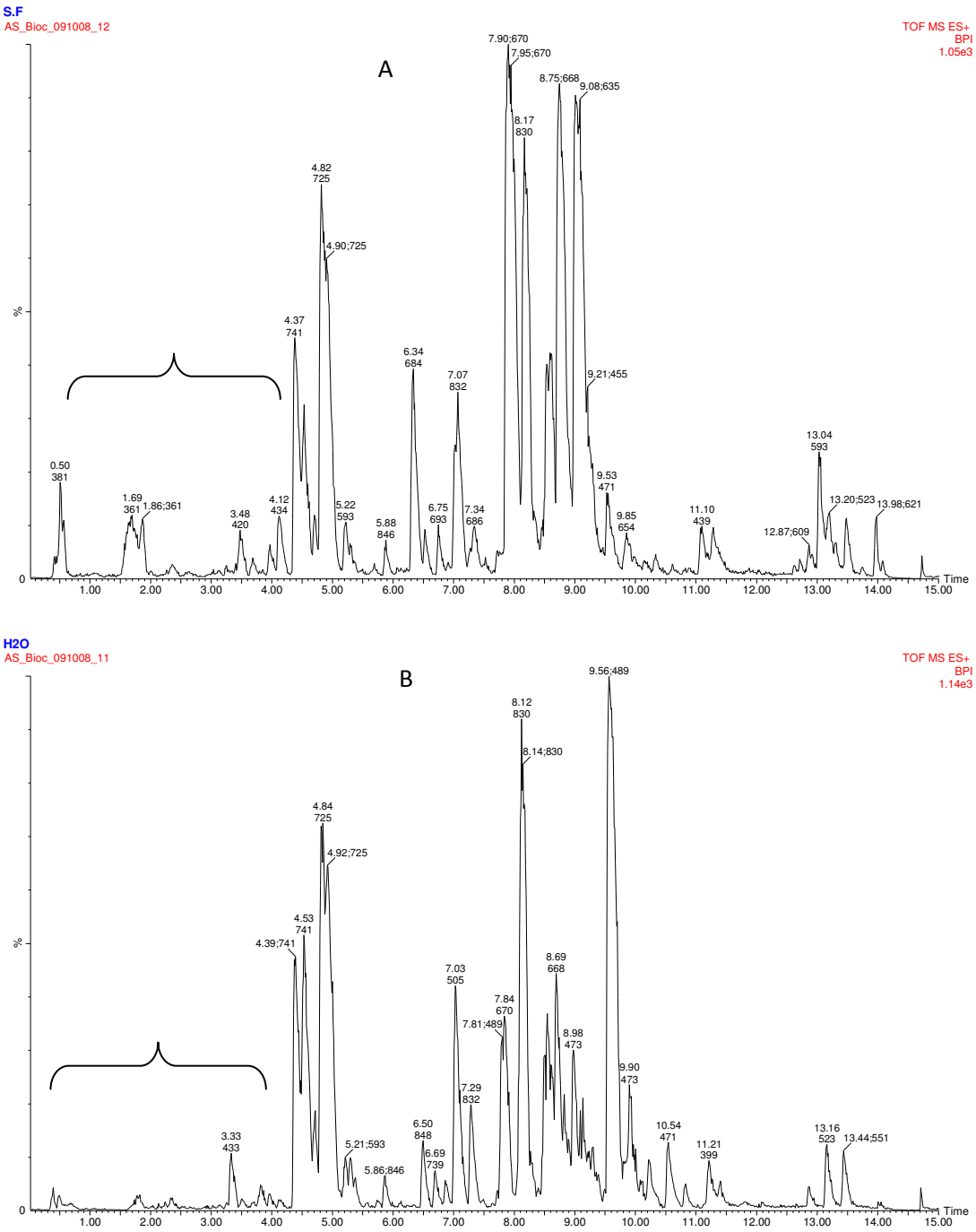


Figure 5.1: Total ion chromatogram (ESI positive) analysis of (A) the methanol and (B) the aqueous extract of *S. frutescens* extracts.

In an attempt to identify the bioactive compounds in the methanol extract of *S. frutescens*, the methanol extract was subjected to sequential extractions with organic solvents. This resulted in four extracts — the petroleum ether, ethyl acetate, butanol and residual aqueous extracts. The yields of the respective extracts are shown in Table 5.1. The extracts were analyzed by LC-MS in an attempt to isolate single compounds and the total ion chromatograms are shown in Figure 5.2.A-5.2.D.

Table 5.1: Dried plant extracts obtained by the sequential extractions of 5 ml methanol extract of *S. frutescens* [53.43 mg/ml]. The extracts were resuspended in 1 ml ethylene glycol.

Extraction solvent	Dried extract (total)(mg)	Concentration mg/ml
Petroleum ether	52.3	52.3
Ethyl acetate	58.1	58.1
Butanol	25.2	25.2
residual water phase	18.6	18.6

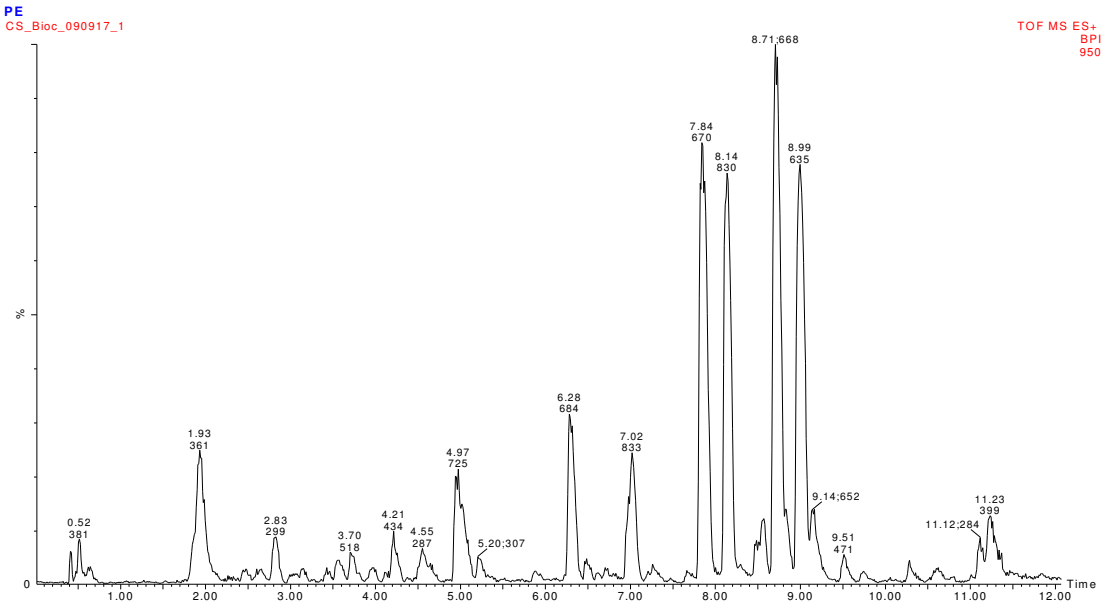


Figure 5.2.A: Total ion chromatogram (ESI positive) analysis of the petroleum ether extract of *S. frutescens*.

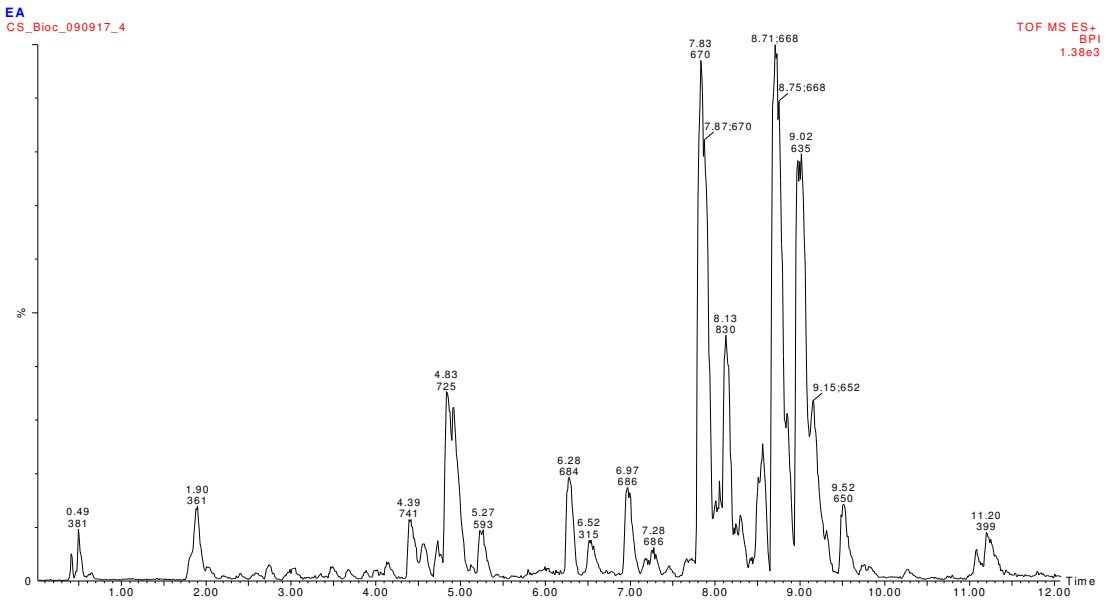


Figure 5.2.B: Total ion chromatogram (ESI positive) analysis of the ethyl acetate extract of *S. frutescens*.

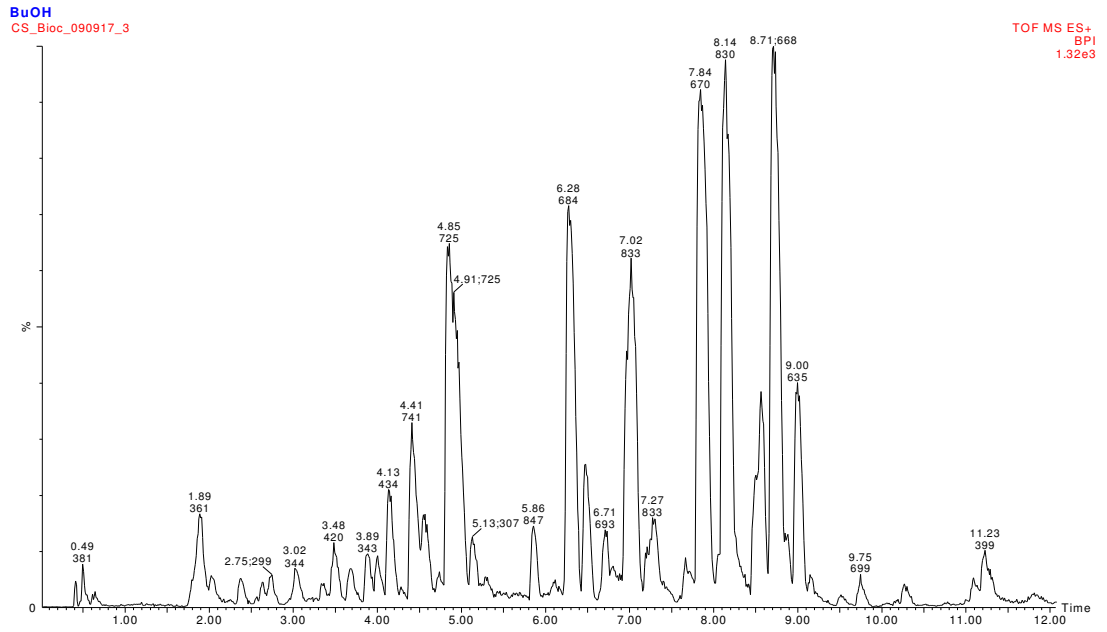


Figure 5.2.C: Total ion chromatogram (ESI positive) analysis of the butanol extract of *S. frutescens*.

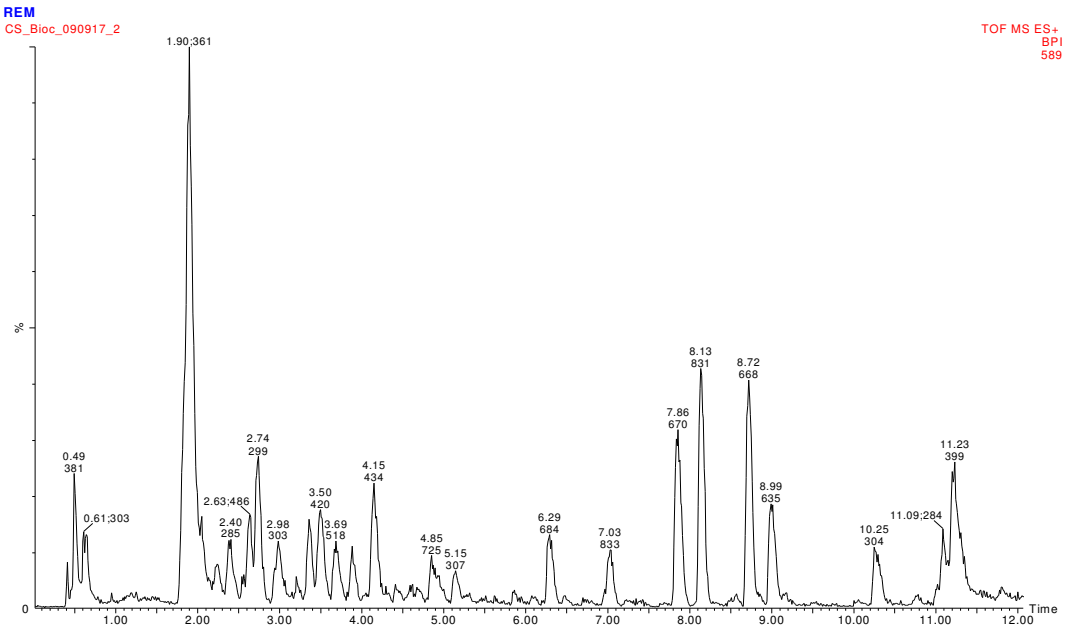


Figure 5.2.D: Total ion chromatogram (ESI positive) analysis of the residual aqueous extract of *S. frutescens*.

The LC-MS analysis revealed that the petroleum ether, ethyl acetate and butanol extracts contained similar compounds, although the butanol extract contained more hydrophilic compounds compared to the petroleum ether and ethyl acetate extracts. The residual aqueous extract appeared to contain very few compounds, although a strong signal was observed at 1.90 minutes, which indicates a relatively small, hydrophilic compound with a molecular mass of 361. Although the chromatograms of these extracts did give some indication of the hydrophobic characteristics of the compounds present, the compounds were not restricted to certain phases. Therefore, the methanol extract of *S. frutescens* was also subjected to fractionation by HPLC.

The fractionation of the methanol extract of *S. frutescens* resulted in five fractions (Table 5.2). In addition, fractions were also collected and protected from light throughout the isolation procedure to determine if the exposure to light affected the bioactivity of the compounds within the fractions. The total ion chromatograms of the isolated fractions are shown in Figure 5.3.A-5.3.E.

Table 5.2: Dried *Sutherlandia frutescens* [53.42 mg/ml] fractionated by HPLC.

Fraction	Dried extract (total) (mg)	Concentration mg/ml
Fraction 1	42	21
Fraction 2	10	5
Fraction 3	6.4	3.2
Fraction 4	15.5	7.75
Fraction 5	11	5.5

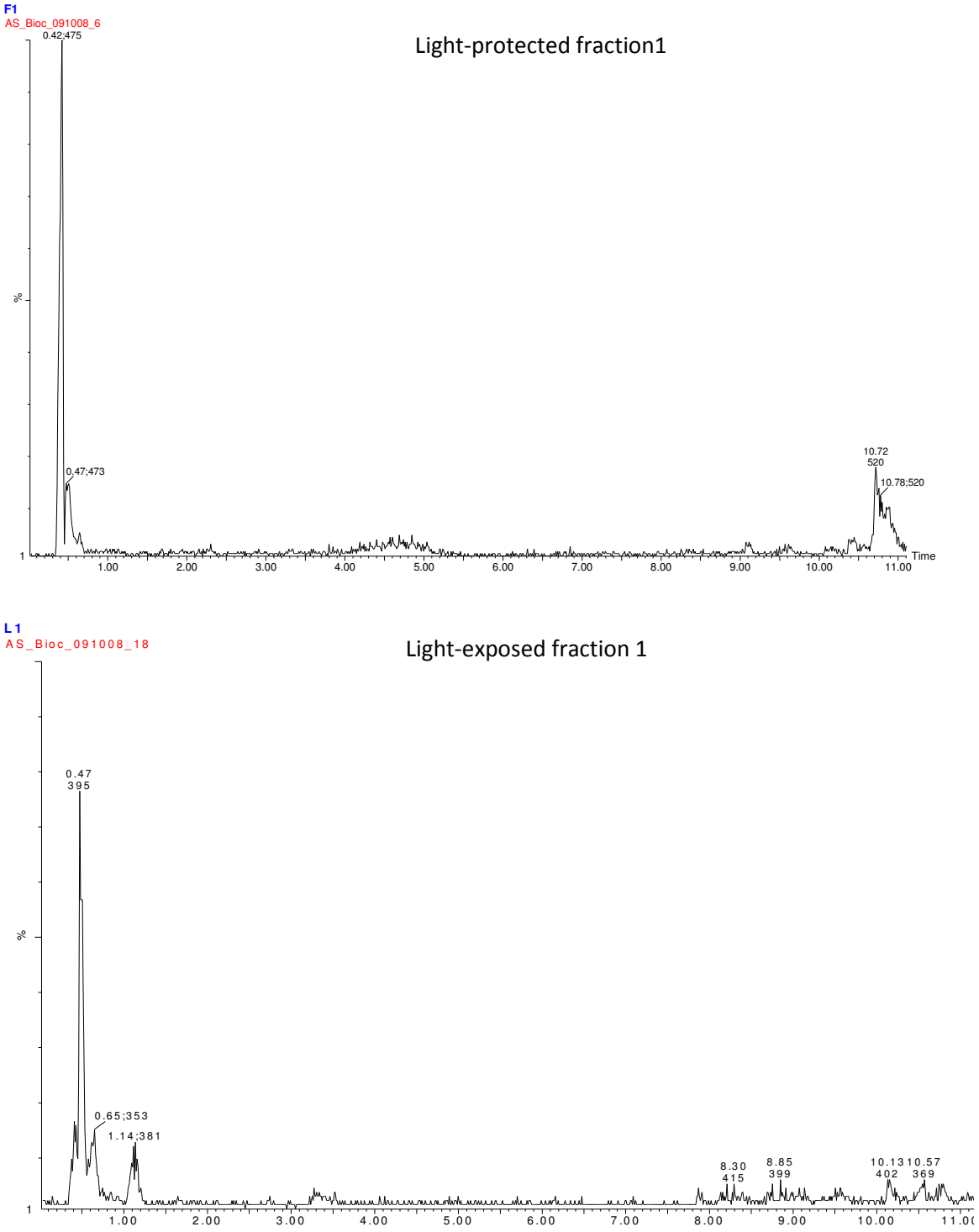


Figure 5.3.A: Total ion chromatogram (ESI positive) analysis of fraction 1 of *S. frutescens* extracts. The light exposed and light protected fractions are indicated on the chromatograms.

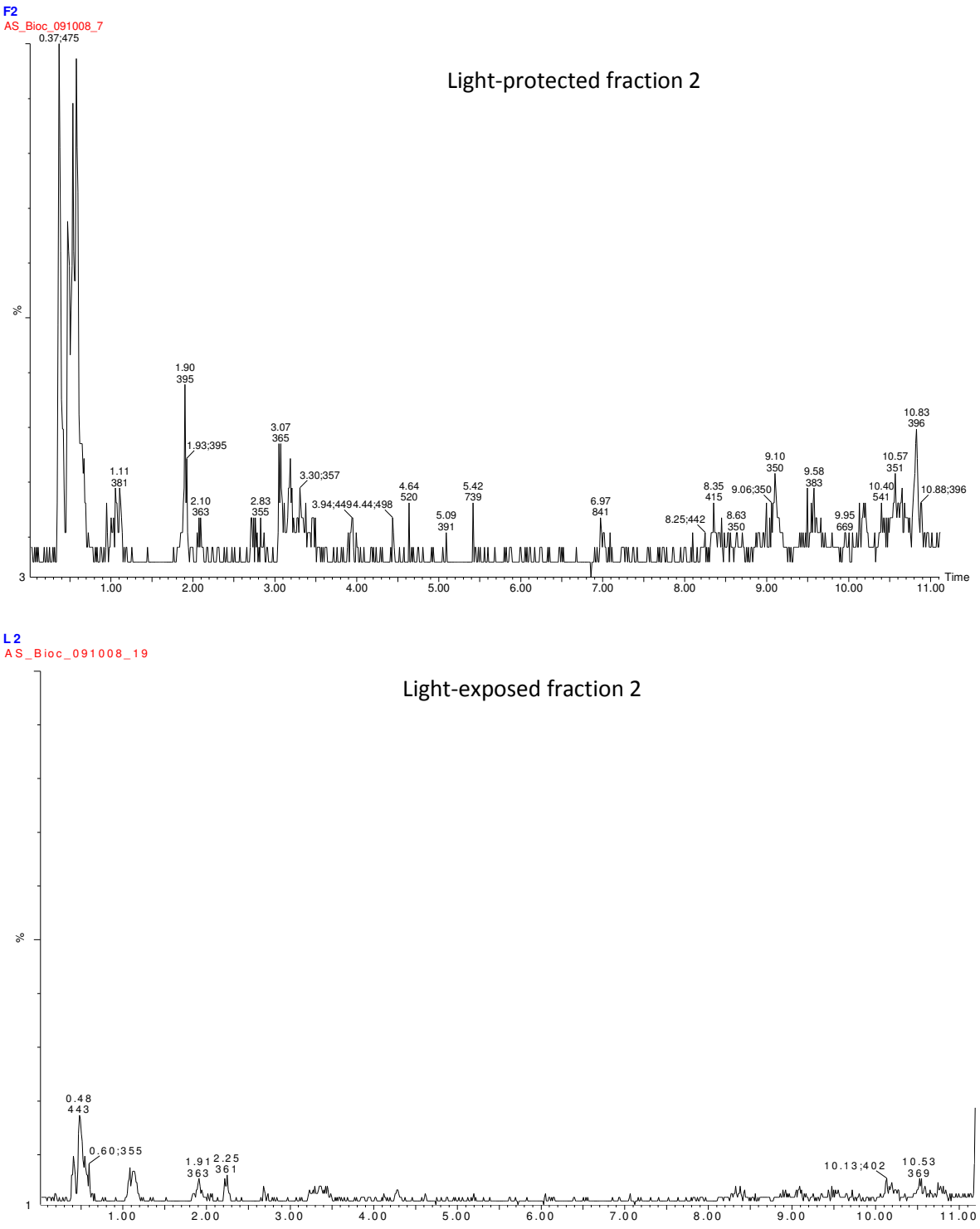


Figure 5.3.B: Total ion chromatogram (ESI positive) analysis of fraction 2 of *S. frutescens* extracts. The light exposed and light protected fractions are indicated on the chromatograms.

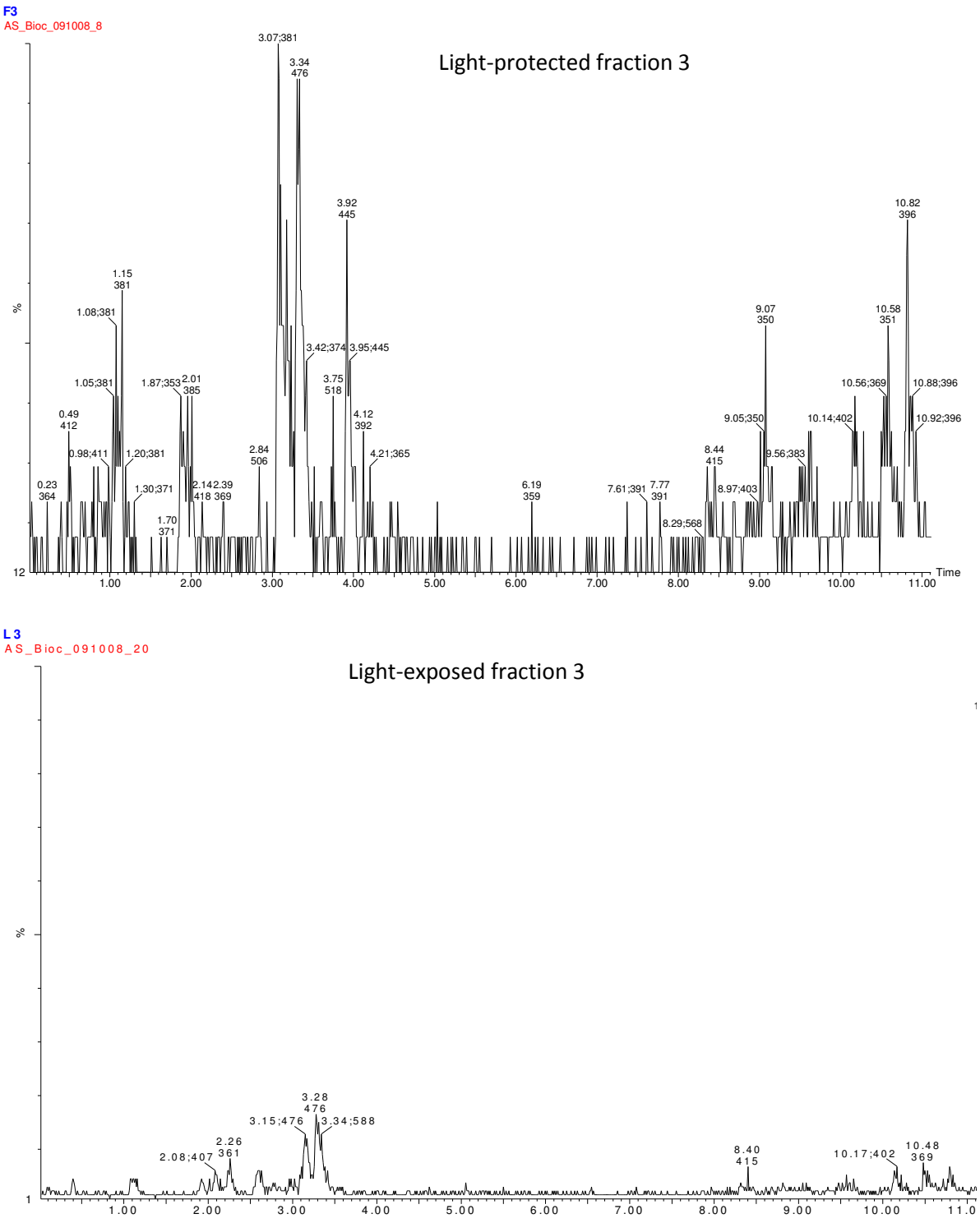


Figure 5.3.C: Total ion chromatogram (ESI positive) analysis of fraction 3 of *S. frutescens* extracts. The light exposed and light protected fractions are indicated on the chromatograms.

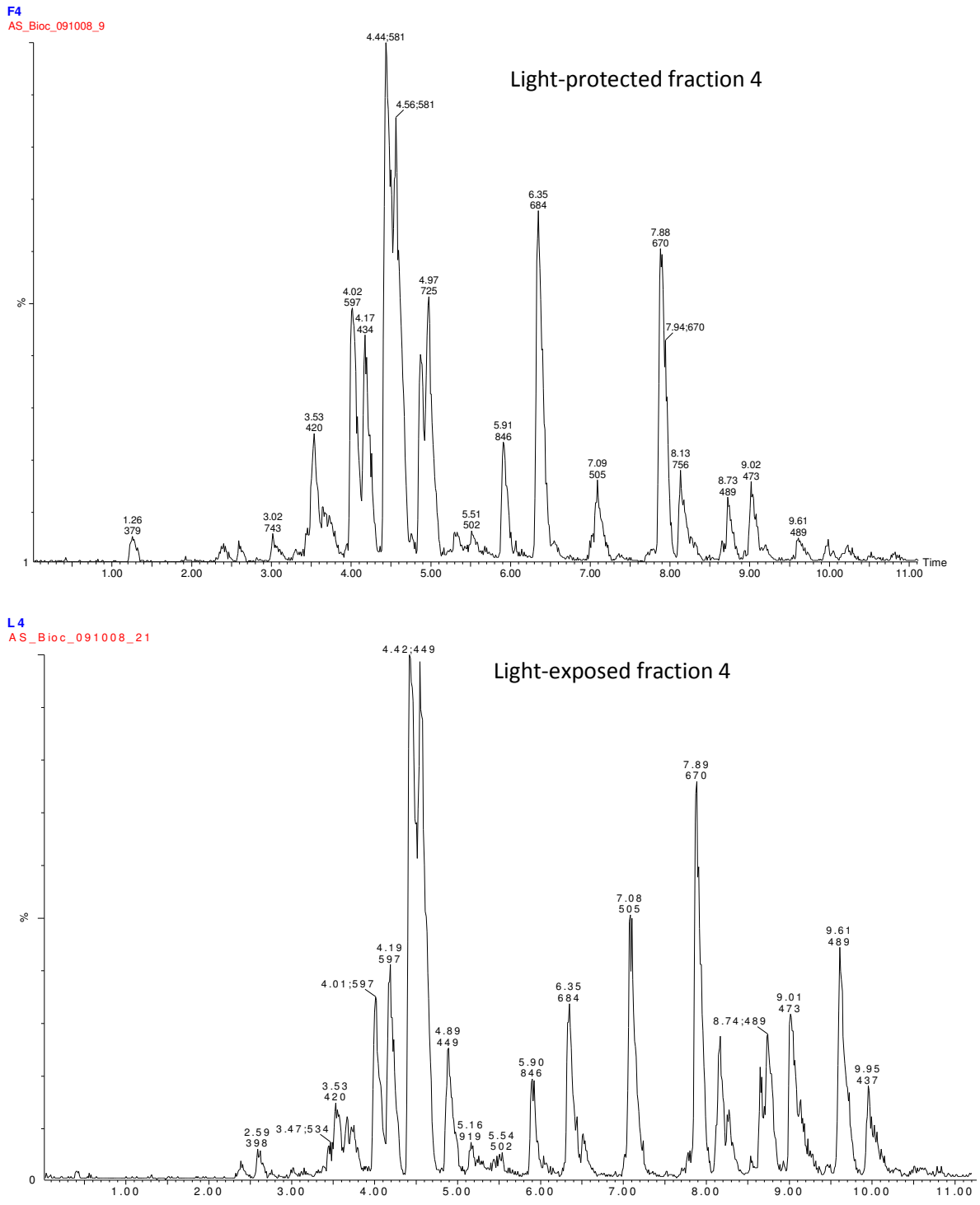


Figure 5.3.D: Total ion chromatogram (ESI positive) analysis of fraction 4 of *S. frutescens* extracts. The light exposed and light protected fractions are indicated on the chromatograms.

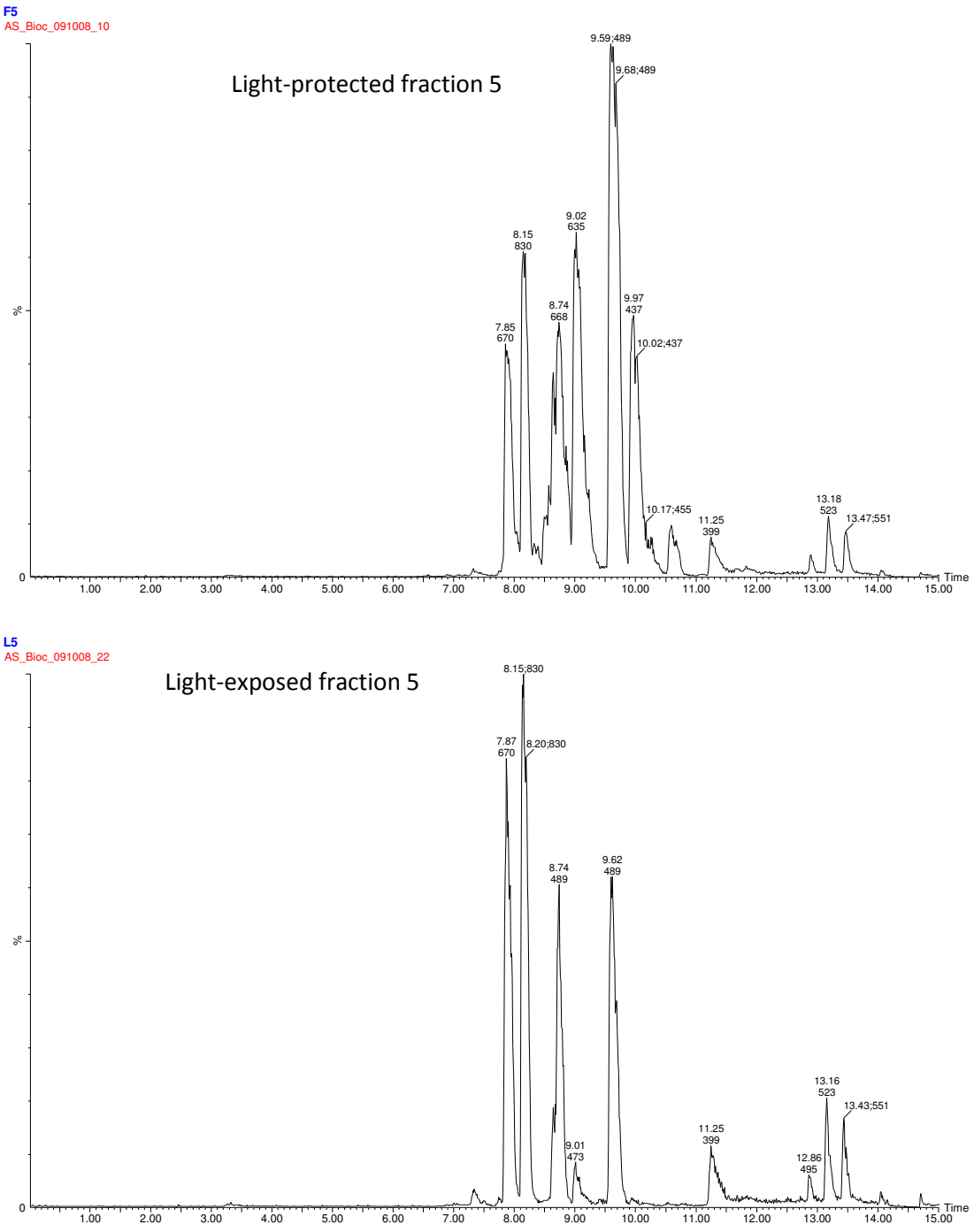


Figure 5.3.E: Total ion chromatogram (ESI positive) analysis of fraction 5 of *S. frutescens* extracts. The light exposed and light protected fractions are indicated on the chromatograms.

Fractions 1-4 appear to be significantly affected by the exposure to light, exhibiting a marked reduction in the signal produced by the compounds present. This may indicate that the compounds are degraded upon exposure to light.

The exposure to light appeared not to affect the compounds present in fraction 5 to the same extent as the compounds in the other fractions. Although the concentrations of the various compounds present in the fractions cannot be determined from these chromatograms, fraction 5 appears to be the least affected by the exposure to light. This indicates that the majority of the compounds in fraction 5 are relatively stable.

Since no single compound could be identified after the sequential extraction and fractionation of the methanol extract of *S. frutescens*, the extracts and the fractions were scanned for the presence of flavonoid compounds and SU1. The analysis included five flavonoid standards – vitexin, orientin, rutin, iso-orientin and iso-vitexin, which have been identified in rooibos [Perold, MSc thesis, 2009], as well as SU1 which has been isolated from *S. frutescens* [Fu *et al* (2008)]. LC-MS analysis confirmed the presence of vitexin, orientin, rutin, and SU1, but no iso-orientin or iso-vitexin was detected (Figure 5.4. A-5.4.D). The concentrations of these compounds in each fraction and extract have been summarized in Table 5.3.

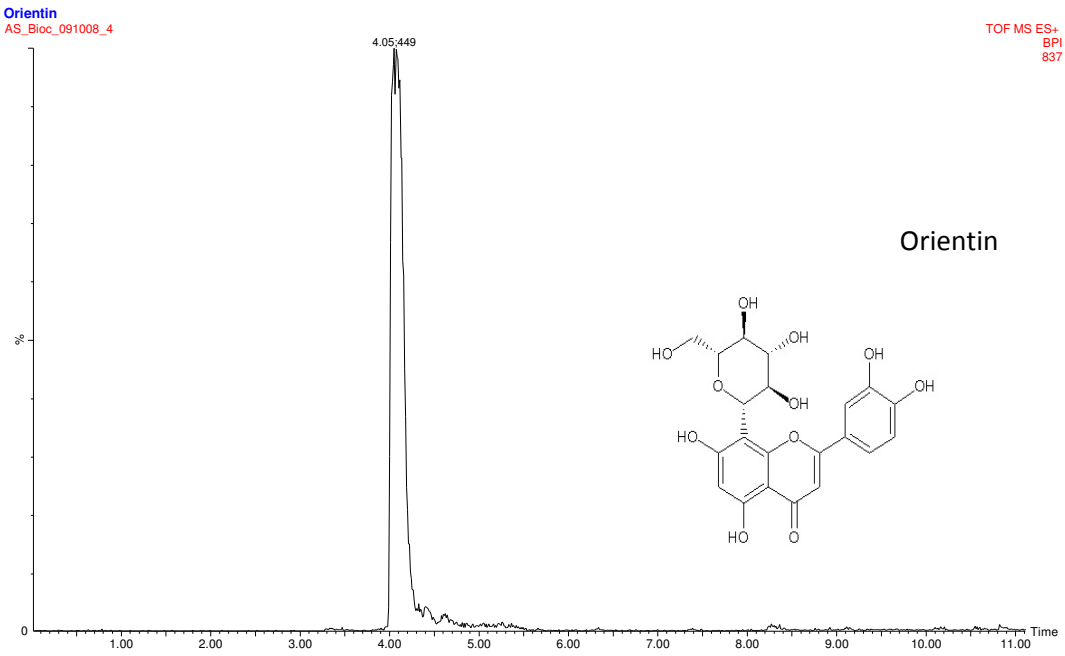


Figure 5.4.A: LC-MS chromatogram of orientin standard.

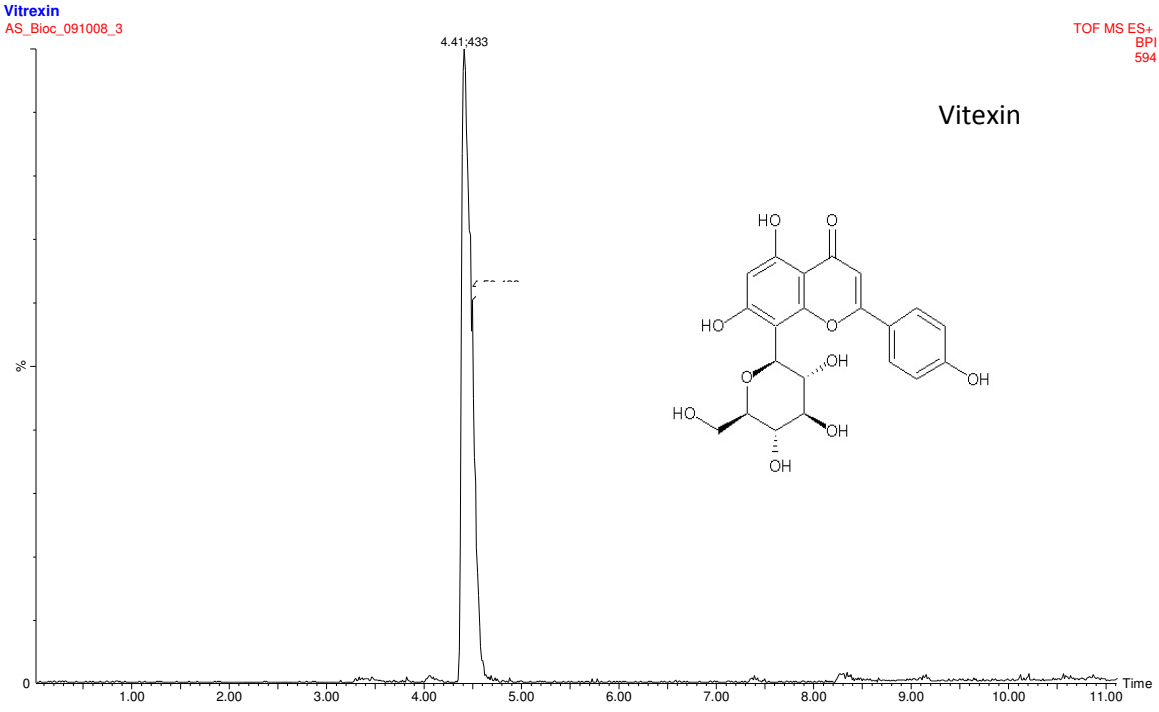


Figure 5.4.B: LC-MS chromatogram of vitexin standard.

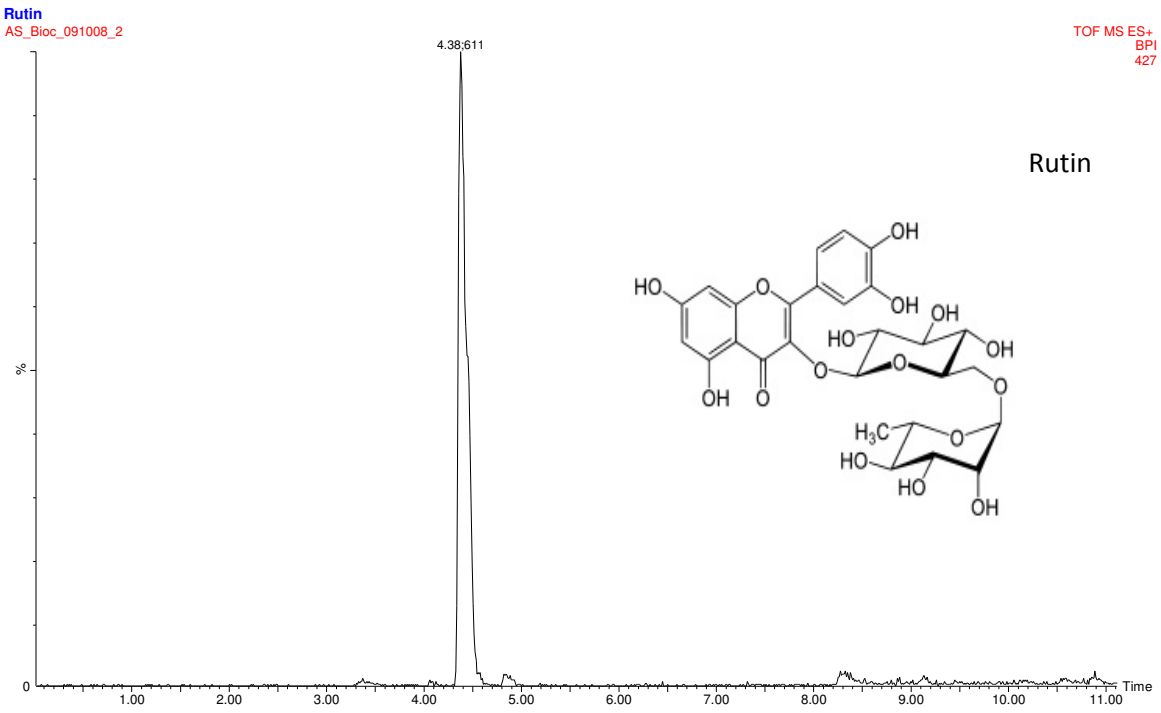


Figure 5.4.C: LC-MS chromatogram of rutin standard.

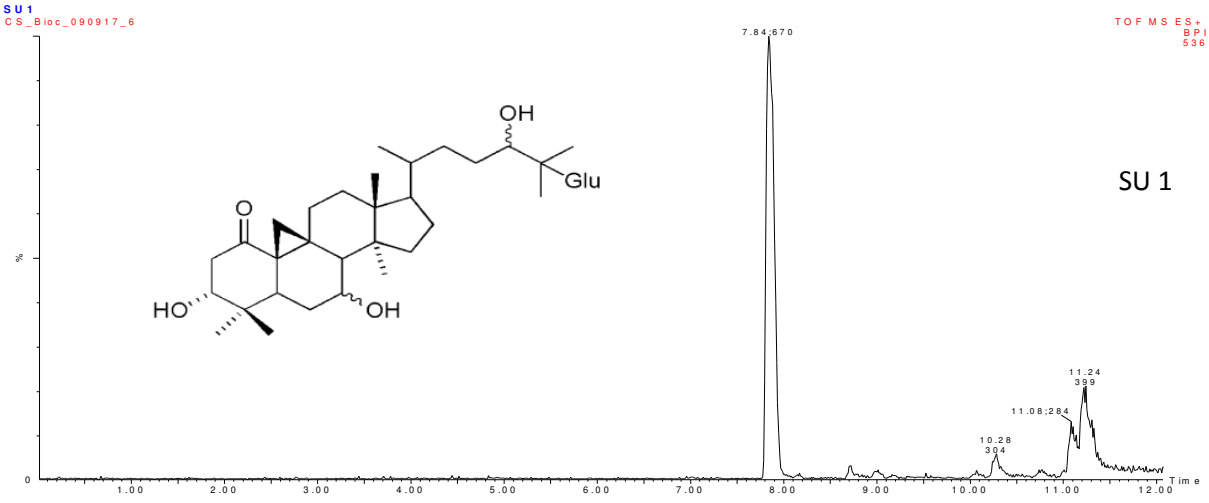


Figure 5.4.D: LC-MS chromatogram of SU1 standard.

Table 5.3: Flavonoids and SU1 identified in the extracts and fractions of *S. frutescens*. The concentration (nM) and the extract and fractions in which the compounds were identified are shown.

Extracts/ Fraction	Vitexin (nM)	Orientin (nM)	Rutin (nM)	SU1 (nM)
Methanol extracts of *S. frutescens*	11.35	5.48	14.56	1.71
Aqueous extracts of *S. frutescens*	0	0	19.54	0.61
Petroleum ether	0	0.37	2.33	0.43
Ethyl acetate	0	0	2.18	0.56
Butanol	0	0	5.68	0.62
Residual aqueous	11.25	4.95	4.37	0.13
Light Fraction 1	0.19	0.70	0	0
Light Fraction 2	0.39	0.89	0	0
Light Fraction 3	0.77	0.88	0	0
Light Fraction 4	8.85	1.38	1.98	0.31
Light Fraction 5	0.38	0	0	0.90
Fraction 1	1.92	2.74	0	0
Fraction 2	6.81	0	0	0
Fraction 3	1.02	0.89	0	0
Fraction 4	1.10	1.21	6.47	0.36
Fraction 5	0.41	0.38	0	0.85

Although rutin was present in the petroleum ether, ethyl acetate, butanol and residual aqueous extract, with the highest concentration present in the butanol extract, this was not

the case for vitexin and orientin. Orientin was present at a low concentration in the petroleum ether extract, whereas vitexin was not present in the petroleum ether, ethyl acetate, butanol or residual aqueous extract. SU1, however, was present in all the extracts. The highest concentration of SU1 was present in the butanol extract.

The concentrations of the flavonoids and SU1 present in the light-protected fractions and the light-exposed fractions differ significantly. It appears that the light-exposed fractions exhibit a marked decrease in flavonoid concentrations, although the SU1 appears to be stable. The highest concentration of vitexin is present in fraction 4 of the light-exposed fractions and in fraction 2 in the light-protected fractions. The highest concentration of orientin is present in fraction 1 of the light-protected fractions, with no significant presence in the light-exposed fractions. This indicates that this compound is labile and may be degraded upon exposure to light. In the fractions, rutin was present in fraction 4 at a concentration 3 fold greater than the concentration of rutin in the light-exposed fractions. This may indicate that rutin is also labile and may degrade upon exposure to light.

Although the flavonoids and SU1 were detected in the different extracts and fractions, the highest concentrations of orientin, vitexin and SU1 were present in the methanol extract of *S. frutescens*. Although the methanol extract of *S. frutescens* did contain a relatively high concentration of rutin, a greater concentration of rutin was present in the aqueous extract.

The confirmed presence of the flavonoids and SU1 does not necessarily indicate the bioactivity of the different extracts and fractions. Their bioactivity was therefore assayed to determine if the flavonoids inhibited CYP11B1 and could ultimately influence glucocorticoid production. Spectral binding assays in ovine adrenal mitochondria and substrate conversion assays in COS1 cells were carried out.

5.3.2 Influence of the methanol extract of *S. frutescens* on substrate binding

The P450 content of the ovine adrenal mitochondria was determined by using the carbon monoxide-induced difference spectra and calculating the P450 concentration using the molar extinction coefficient (ε) of 91 cm^{-1} mM^{-1} [Omura & Sato (1964)]. Even in the

presence of a strong reducing agent, the reduction of cytochrome P450 enzymes is a slow reaction and must be allowed to develop. The sodium dithionate reduction of the carbon monoxide induced spectrum of ovine adrenal mitochondrial P450 after 10 minutes is shown in Figure 5.5.

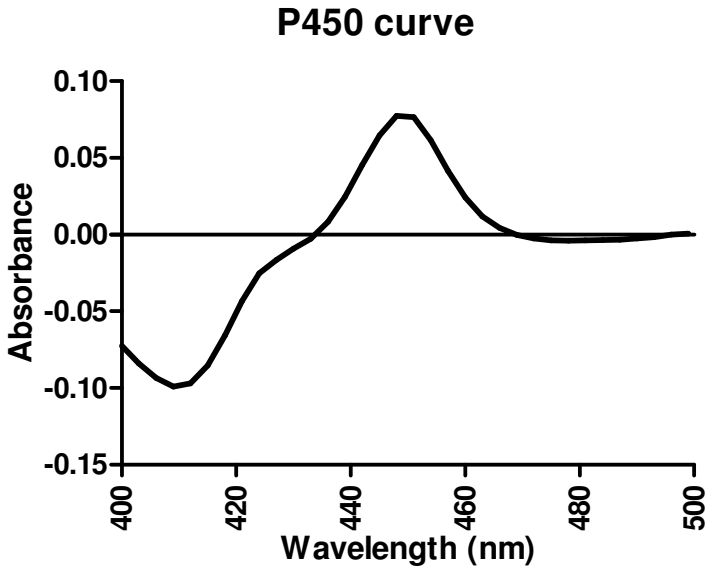


Figure 5.5: Sodium dithionate reduced carbon monoxide-induced difference spectrum of ovine adrenal mitochondria P450. [P450], 0.88 μM; [Protein], 0.76 mg protein/mg mitochondrial powder.

Substrate-induced difference spectra are produced when substrates bind to P450 enzymes. The binding of DOC and deoxycortisol yielded type I difference spectra with a peak maximum at 390 nm and a peak minimum at 420 nm when assayed in ovine adrenal mitochondria (Figure 5.6).

The biological activity of the methanol extract of *S. frutescens* was assayed by investigating the influence of these extracts on the ability of DOC and deoxycortisol to bind to CYP11B1 in ovine adrenal mitochondria. Substrate binding assays carried out in the presence of the *S. frutescens* extract would indicate if compounds present in *S. frutescens* could interfere with the binding of the natural substrates, DOC and deoxycortisol, thus preventing product formation *in vivo*.

In the presence of *S. frutescens* extract a reduction in amplitude between the peak maximum at 390 nm and the peak minimum at 420 nm was observed (Figure 5.6). The difference in the amplitudes of the two spectra was used to calculate the percentage inhibition (Equation 2).

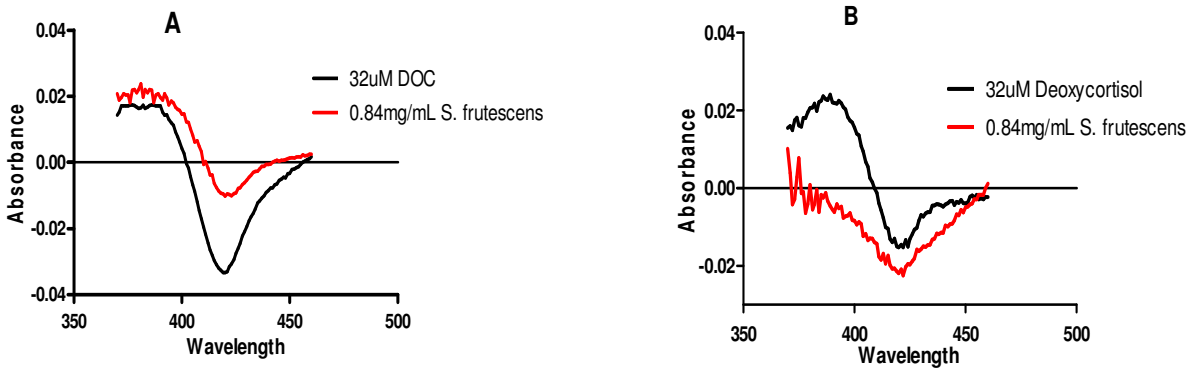


Figure 5.6: Type I substrate-induced difference spectra assayed in ovine adrenal mitochondria. (A) DOC-induced difference spectrum, [DOC], 32 µM; (B) deoxycortisol-induced difference spectrum, [deoxycortisol], 32 µM; [P450], 0.88 µM; [Protein], 0.76 mg protein/mg mitochondrial powder and [*S. frutescens*], 0.84 mg/ml. Results are representative of three independent experiments and are shown as the mean of triplicate experimental values.

The influence of *S. frutescens* extracts, 0.21 mg/ml, 0.42 mg/ml and 0.84 mg/ml, on DOC and deoxycortisol binding (Figure 5.7 and 5.8) was subsequently assayed. Since the degree of inhibition by the methanol extract differed with regards to DOC and deoxycortisol, the inhibition was investigated at two different substrate concentrations, 8 and 32 µM, respectively. Although the extract concentrations assayed exhibited inhibition of the binding of the steroid substrates to CYP11B1 significantly ($P<0.01$), a 50 % inhibition of deoxycortisol binding was observed at 32 µM in the presence of 0.21 mg/ml extract (Figure 5.7). However, at the same steroid concentration, 0.21 mg/ml extract was unable to inhibit the binding of DOC to the enzyme. At a concentration of 0.84 mg/ml, the extract exhibited 35 % inhibition ($P<0.01$) of DOC binding with no further inhibition at the higher extract concentration (Figure 5.8). It is thus apparent that the inhibition of the binding of deoxycortisol to the enzyme is greater than the inhibition of DOC binding.

At the lower substrate concentration, 8 µM, 100 % inhibition of deoxycortisol binding was obtained at an extract concentration of 0.84 mg/ml, while the inhibition of DOC binding to the enzyme was approximately 40 % ($P<0.01$). Figure 5.9 shows the difference in inhibition of 32 µM deoxycortisol and DOC binding to CYP11B.

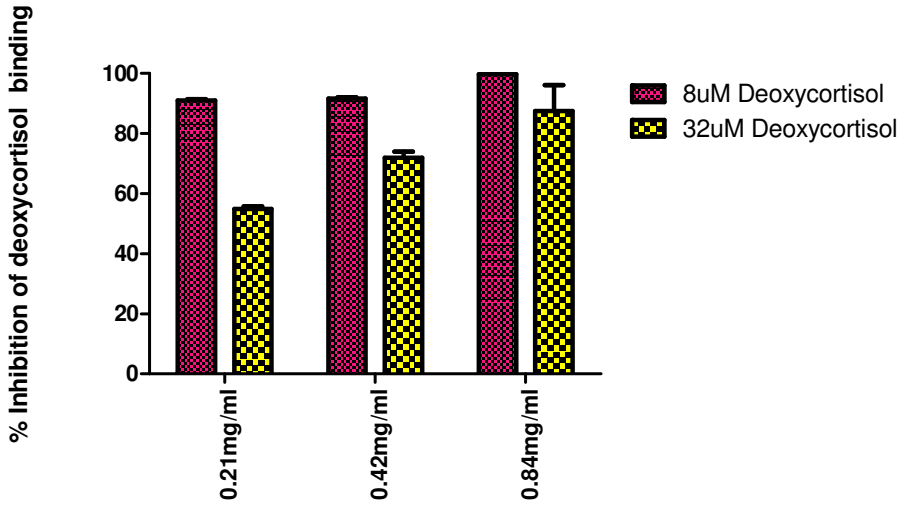


Figure 5.7: Inhibition of deoxycortisol binding to CYP11B1 in ovine adrenal mitochondria in the presence of the methanol extract of *S. frutescens*. [*S. frutescens*], 0.21 mg/ml, 0.42 mg/ml and 0.84 mg/ml; [P450], 0.88µM; [Protein], 0.76 mg protein/mg mitochondrial powder, [Deoxycortisol], 8 µM and 32 µM. Results are representative of three independent experiments. Error bars represent SEM.

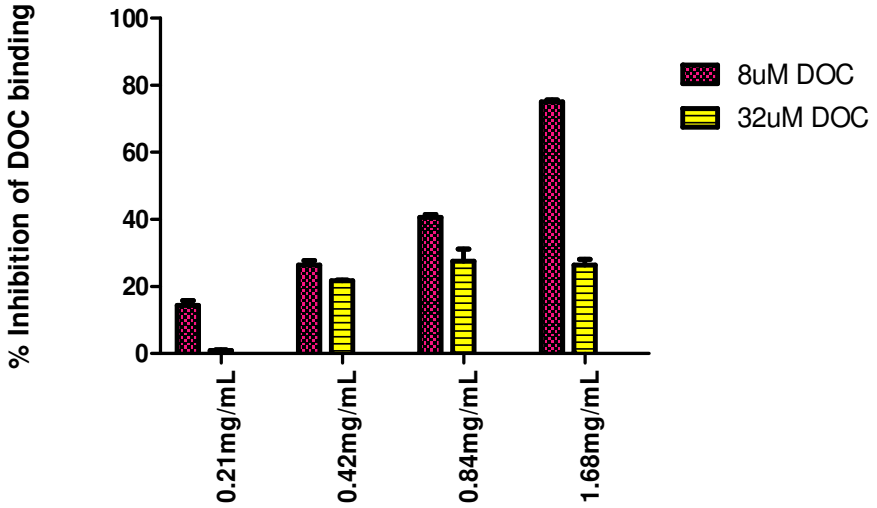


Figure 5.8: Inhibition of DOC binding to CYP11B1 in ovine adrenal mitochondria in the presence of the methanol extract of *S. frutescens*. [*S. frutescens*], 0.21 mg/ml, 0.42 mg/ml and 0.84 mg/ml; [P450], 0.88µM; [Protein], 0.76 mg protein/mg mitochondrial powder, [DOC], 8 µM and 32 µM. Results are representative of three independent experiments. Error bars represent SEM.

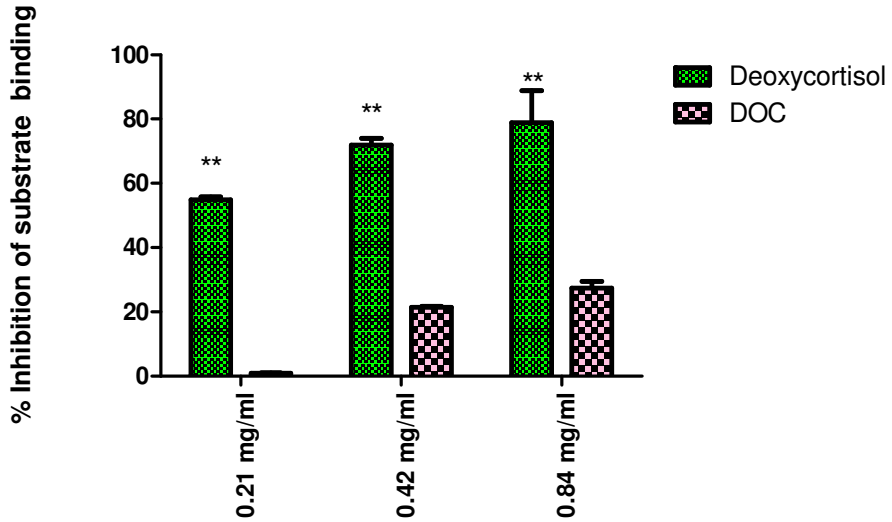


Figure 5.9: Inhibition of deoxycortisol and DOC binding to CYP11B1 in ovine adrenal mitochondria in the presence of the methanol extract of *S. frutescens*. [*S. frutescens*], 0.21 mg/ml, 0.42 mg/ml, 0.84 mg/ml and 1.68 mg/ml; [P450], 0.88µM; [Protein], 0.76 mg protein/mg mitochondrial powder, [Deoxycortisol and DOC], 32µM. Results are representative of three independent experiments. Error bars represent SEM. An unpaired t-test was performed to compare the binding of deoxycortisol to the binding of DOC. ($P<0.05$, **)

The substrate binding data was subsequently plotted generating double-reciprocal plots to visualize the inhibition data and determine the spectral association constant (K_s) for the methanol extract of *S. frutescens* in the presence of DOC and deoxycortisol (Figure 5.10).

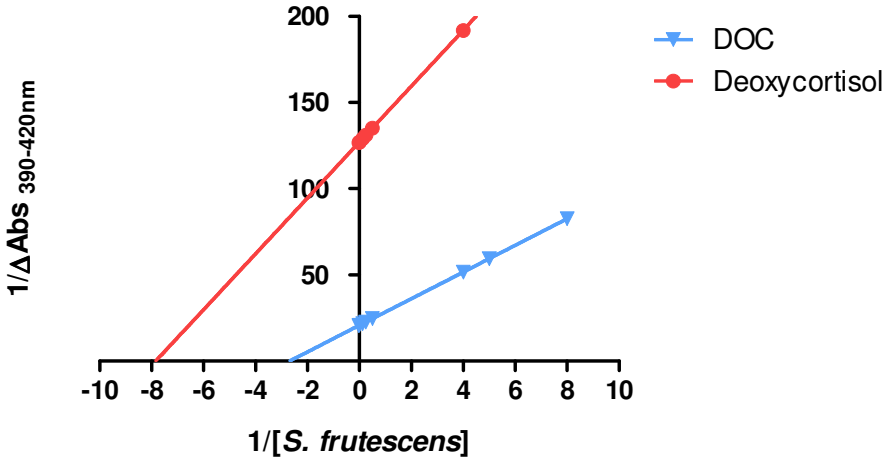


Figure 5.10: Double reciprocal plot of the inhibitory effect of the methanol extract of *S. frutescens* on DOC and deoxycortisol binding in ovine adrenal mitochondria. [*S. frutescens*], 0.21-0.84 mg/ml; [P450], 0.88 µM; [DOC], 32 µM; [Deoxycortisol], 32 µM. Results are representative of three independent experiments.

The K_s for the methanol extract of *S. frutescens* in the presence of DOC and deoxycortisol are 0.37 mg/ml and 0.13 mg/ml, respectively. Thus 0.13 mg/ml of the methanol extract of *S. frutescens* is required to inhibit the binding of deoxycortisol to CYP11B1 by 50 %, compared to 0.37 mg/ml methanol extract of *S. frutescens* required to elicit a 50 % inhibition of DOC binding to CYP11B1. This data correlates with the findings shown in Figure 5.7 and 5.8, confirming that deoxycortisol binding is more sensitive to the presence of the methanol extract of *S. frutescens* than the binding of DOC to CYP11B1 in adrenal mitochondria.

5.3.3 Influence of the methanol extracts of *S. frutescens* on substrate conversion

Although the presence of the methanol *S. frutescens* extract inhibited the binding of DOC and deoxycortisol to ovine adrenal CYP11B1, the substrate-induced difference spectra do not indicate whether the conversion of DOC and deoxycortisol by CYP11B1 is affected. In addition, the inhibitory effect elicited by the methanol *S. frutescens* extract on the binding of DOC and deoxycortisol, respectively, varied considerably. The difference in the extent of inhibition of substrate binding in the presence of *S. frutescens* may also imply that a different type of inhibition occurs in the presence of DOC and deoxycortisol. Consequently, CYP11B1 was expressed in COS1 cells and the conversion of DOC and deoxycortisol to corticosterone and cortisol, respectively, was investigated in the absence and presence of the methanol *S. frutescens* extract. The steroid content of the samples collected from the conversion assays were analyzed and quantified using LC-MS.

5.3.3.1 DNA isolation

The isolation of the plasmid constructs required for the transfection procedure was performed as described in section 5.2.8.1. The results are shown in Table 5.4. The separation of the plasmids by gel electrophoresis, using a 0.7 % agarose gel, confirms that the majority of the plasmid DNA constructs is super coiled and 260/280 nm ratios imply that the plasmids consist of pure DNA. The top band indicates DNA nicked during the preparation of the plasmid and the lower band shows the supercoiled closed circular DNA which is essential for maintaining high transfection efficiency (Figure 5.11).

Table 5.4: Plasmid yield, final plasmid concentration and plasmid purity of the plasmid constructs isolated from 100 ml transformed *E. coli*.

Plasmid construct	Yield (µg)	260/280 nm	Final concentration (µg/µl)
Baboon CYP11B1/pTarget	108.6	1.897	0.543
Human ADX/pTarget	472.4	1.797	2.362
Angora CYP17/pVector	440.0	1.815	2.200
pCI-neo	572.6	1.856	2.863

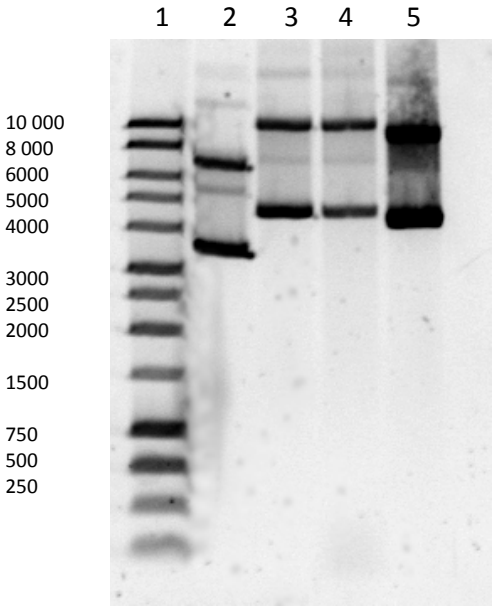
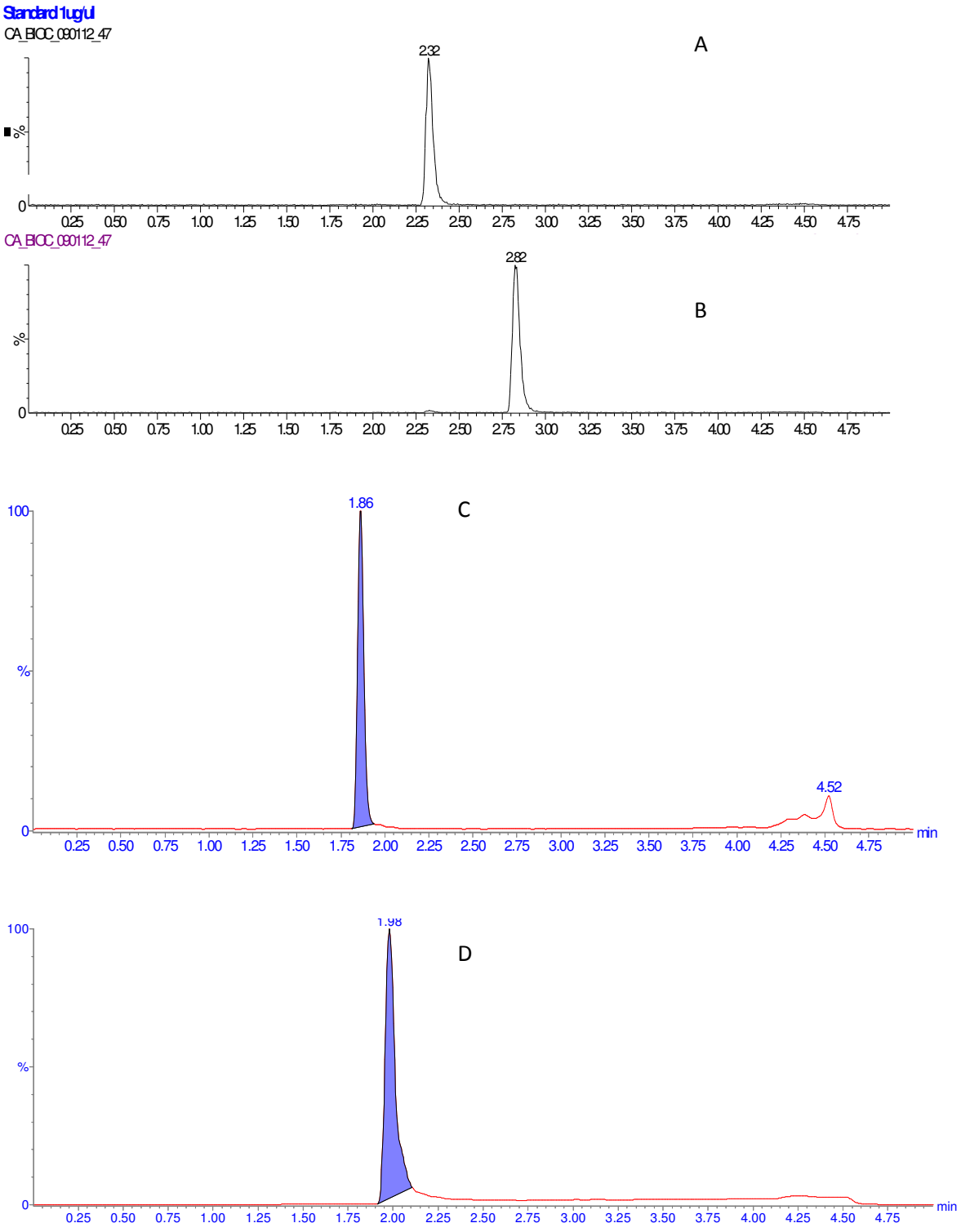


Figure 5.11: Gel electrophoresis of isolated plasmid constructs. Lane 1, 1 Kb ladder (Promega); Lane 2, pCI-neo; Lane 3, angora CYP17/pVector; Lane 4, baboon CYP11B1/pTarget and Lane 5, human ADX/pTarget. Every well contained 150 ng of DNA. A 0.7 % agarose gel was used and the bands were visualized with Sybr gold.

5.3.3.2 LC-MS analysis of steroids

Standard curves for steroids were constructed by plotting the peak ratios of the steroids against the steroid concentrations, using concentrations ranging from 1 ng/ml - 0.01 ng/ml. The resulting calibration curves were linear with regression correlation

coefficients above 0.99. An example of the separation of the steroid standards, on a C18 column, is shown in Figure 5.12.



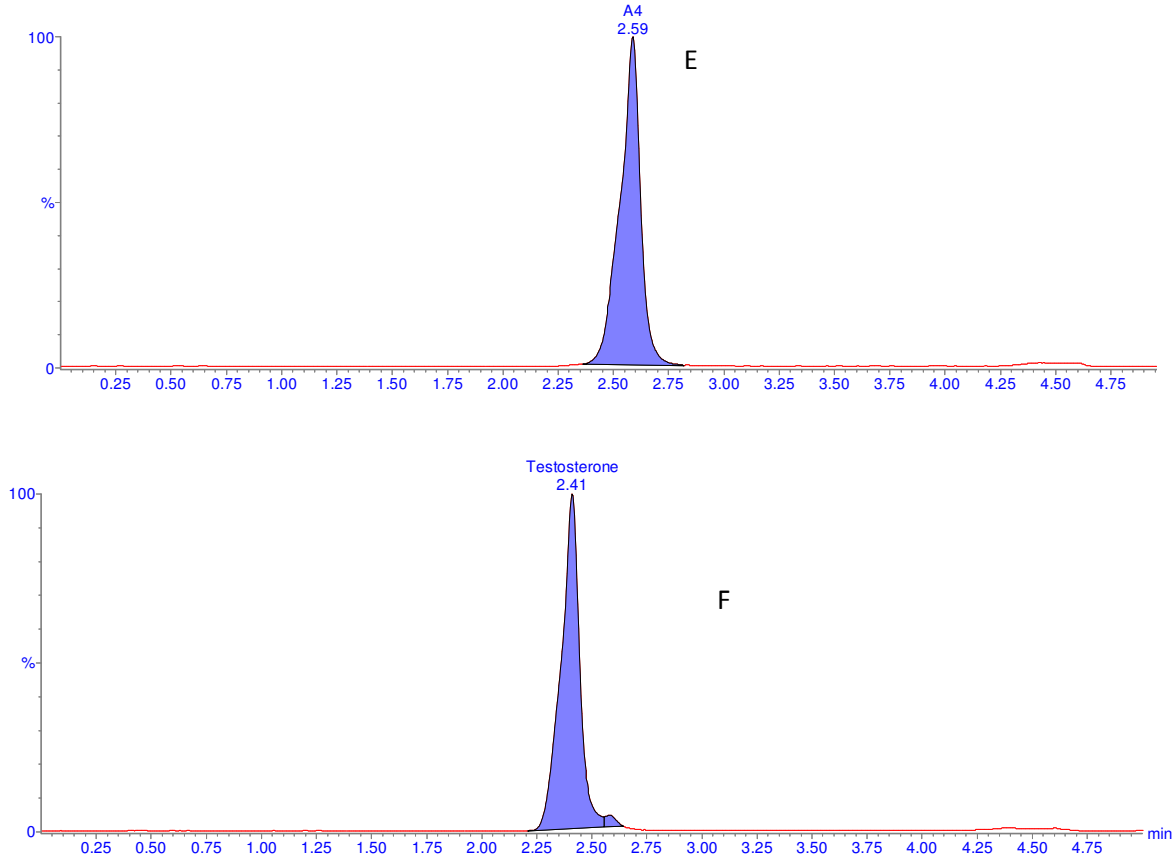


Figure 5.12: LC-MS chromatograms of steroid standards. (A) corticosterone, *m/z* 347, retention time, 2.32 minutes, (B) DOC, *m/z* 363.3, eluting at 2.82 minutes, (C) cortisol, *m/z* 362, retention time, 1.86 minutes, (D) deoxycortisol, *m/z* 347, retention time, 1.98 minutes, (E) testosterone, *m/z* 289, retention time, 2.41 minutes and (F) androstenedione, *m/z* 286, retention time, 2.59 minutes.

5.3.3.3 Substrate conversion assays

The influence of methanol extracts of *S. frutescens* on the conversion of DOC and deoxycortisol to corticosterone and cortisol, respectively, by CYP11B1 was investigated in COS1 cells. COS1 cells were transfected with CYP11B1 and ADX. CYP11B1 requires the presence of ADX for electron transport from the NADPH cofactor. The conversion of DOC to corticosterone in the absence of extracts was assayed over a 24 hour period and is shown in Figure 5.13. The controls used for this experiment and downstream conversion assays were COS1 cells co-transfected with the angora CYP17 and pCIneo plasmid constructs to assess transfection efficacy and pCIneo to ensure specificity of the

enzyme and to confirm that the results obtained from this experiment are due to the transfected COS1 cells.

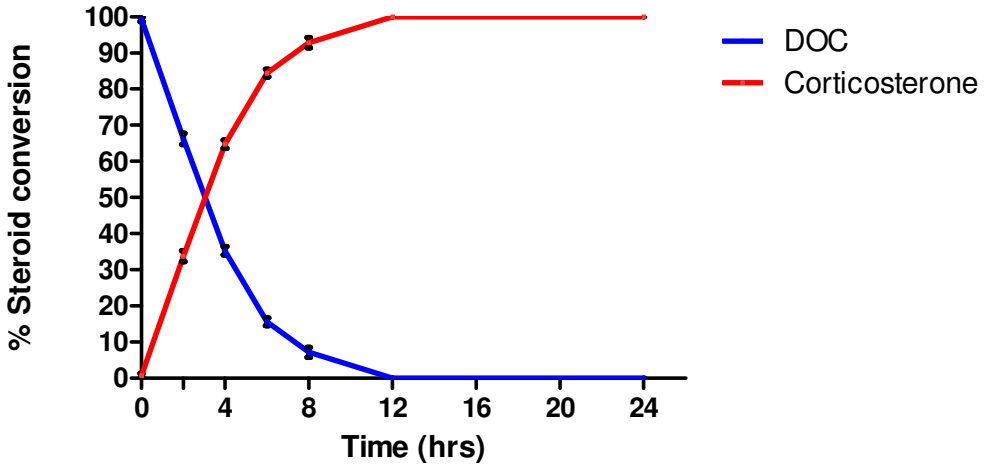


Figure 5.13: Conversion of DOC to corticosterone in COS1 cells expressing baboon CYP11B1. Initial [DOC], 1 µM. Results are presented as the mean of three experimental values. Results are representative of three independent experiments. Error bars represent SEM.

From Figure 5.13 it is clear that 1 µM of DOC is completely converted to corticosterone by CYP11B1 over a period of 12 hours. Based on this data, steroid samples were taken for analysis 8 hours after substrate addition in the subsequent conversion assays.

The percentage of corticosterone produced by CYP11B1 from DOC is shown in Figure 5.14. In the absence of the methanol extracts of *S. frutescens*, 100 % DOC conversion to corticosterone was observed at 8 hours. In the presence of 12.2 mg/ml of the extract, corticosterone production was completely inhibited ($P<0.05$). The addition of extracts at concentrations of 2.4 mg/ml and 4.8 mg/ml, elicited a strong inhibitory response and corticosterone production was reduced by approximately 80 % ($P<0.05$) and 91 % ($P<0.05$), respectively.

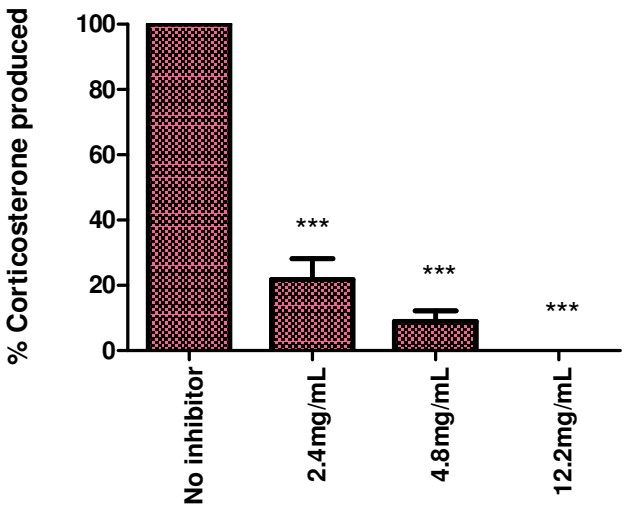


Figure 5.14: Inhibition of the conversion of DOC to corticosterone after 8 hours in COS1 cells expressing baboon CYP11B1. Initial [DOC], 1 µM; [*S. frutescens*], 2.4mg/ml, 4.8mg/ml and 12.2mg/ml. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$, ***).

Similarly, deoxycortisol was completely converted to cortisol by CYP11B1 in the absence of methanol extract in COS1 cells (Figure 5.15). The inhibition of deoxycortisol conversion by *S. frutescens* was similar to the inhibition of DOC conversion although the production of cortisol was not completely inhibited at the highest concentration of the extract assayed. There was a significant difference in the inhibition of deoxycortisol binding elicited in the presence of 12.2 mg/ml extract compared the presence of 2.4 mg/ml and 4.8 mg/ml ($P<0.05$).

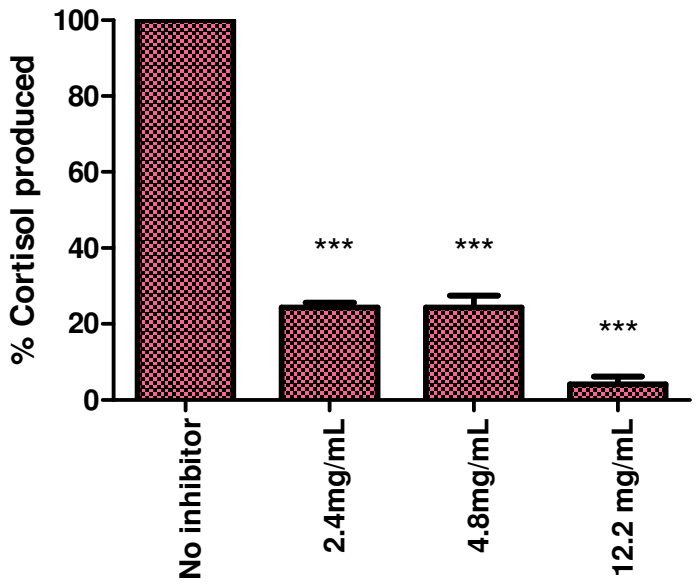


Figure 5.15: Inhibition of the conversion of deoxycortisol to cortisol in COS1 cells expressing baboon CYP11B1 after 8 hours. Initial [deoxycortisol], 1 µM; [*S. frutescens*], 2.4 mg/ml, 4.8 mg/ml and 12.2 mg/ml. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$, ***).

The methanol extract of *S. frutescens* was found to inhibit the binding of DOC and deoxycortisol to CYP11B1 in ovine adrenal mitochondria as well as inhibiting the conversion of DOC and deoxycortisol to cortisone and cortisol, respectively. Although it was clear that the binding of DOC and deoxycortisol to CYP11B1 was inhibited to different degrees, this was not the case for the conversion assays. These assays did not show clear differences in the ability of CYP11B1 to convert DOC and deoxycortisol, thus no unambiguous correlation could be made with regard to the concentration of methanol extract of *S. frutescens* present in the conversion assays. The inhibition of conversion observed in the conversion of deoxycortisol to cortisol at concentrations of 2.4 mg/ml and 4.8 mg/ml, appeared not to be dose dependant and may imply that compounds present in the methanol extract of *S. frutescens* may inhibit or stimulate conversion.

No clear difference in the inhibition of the catalytic activity of baboon CYP11B1 was observed in terms of the different substrates, deoxycortisol and DOC. In addition, it has

been documented that the K_m of baboon CYP11B1 for DOC and deoxycortisol do not differ significantly [Brown (2007)]. This may imply that the catalytic activity of the enzyme is not more sensitive to the presence of the methanol extract of *S. frutescens* with regard to its natural steroid substrates, thus the downstream experiments were performed with DOC as substrate.

In an attempt to identify and isolate compounds within the methanol extract of *S. frutescens* that could be responsible for the inhibition or stimulation of CYP11B1, the methanol extract of *S. frutescens* was subjected to sequential extractions and subjected to fractionation by HPLC. Although the fractions isolated by HPLC and the residual water phase from the sequential extractions could easily be resuspended in de-ionized water after lyophilization, the petroleum ether, ethyl acetate and butanol extractions could not. A problem that needed to be addressed was the choice of the solvent in which the dried solvent extracted material could be resuspended. The solvent would have to be able to dissolve the hydrophobic compounds present in the extracts and would have to be suitable for subsequent experiments, without affecting the viability of the COS1 cells or affecting the ovine adrenal mitochondrial P450 enzymes. A cell viability test was therefore performed.

5.3.4 Cell viability test

COS1 cells possess an endogenous dehydrogenase that converts testosterone to androstenedione. This characteristic was exploited to test the viability of COS1 cells in the presence of various solvents which could possibly be used to re-suspend the dried solvent extractions. Figure 5.16 shows the percentage of androstenedione that was produced by the untransfected COS1 cells in the presence of ethylene glycol, dimethylsulphoxide (DMSO) and methanol. The concentration of solvent added to the cells showed no significant inhibition of androstenedione production ($P>0.05$), and it was thus concluded that the viability of the COS1 cells was not affected. All of these solvents could thus be used to re-suspend the extracts, and any inhibition observed in conversion assays in the presence of the extracts would then be attributed to the extract and not the solvent in which they were resuspended.

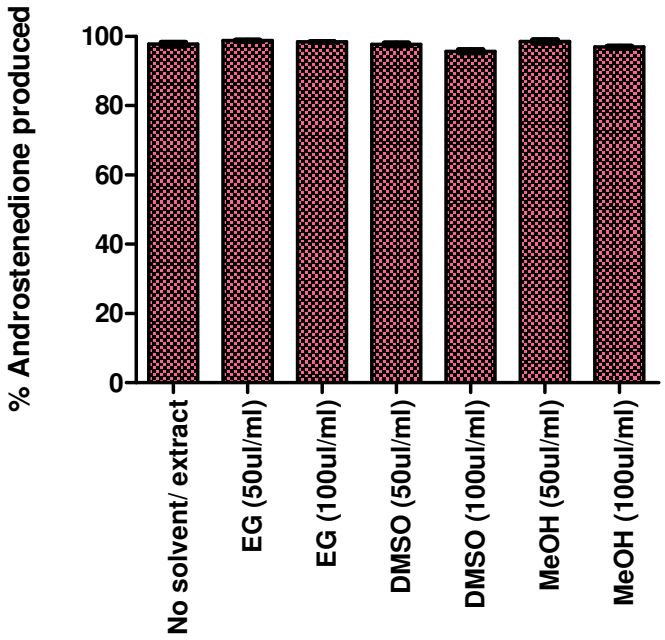


Figure 5.16: Conversion of testosterone to androstenedione in untransfected COS1 cells to determine the effect of respective solvents on the cells. Initial [testosterone], 1µM; EG, ethylene glycol; MeOH, methanol; DMSO, dimethylsulphoxide. Results are representative of one experiment with triplicate data points. Error bars represent SEM.

Although none of the chosen solvents had a significant effect on cell viability, ethylene glycol was used to re-suspend the dried material obtained by the sequential extraction of the methanol extract, since ethylene glycol is known not to interact with P450 enzymes in the mitochondrial suspension.

The viability of the COS1 cells exposed to the methanol extract of *S. frutescens* was assayed in the same manner as described above. The ability of untransfected COS1 cells to convert testosterone to androstenedione was assayed in the absence and presence of the methanol extract of *S .frutescens* (Figure 5.17).

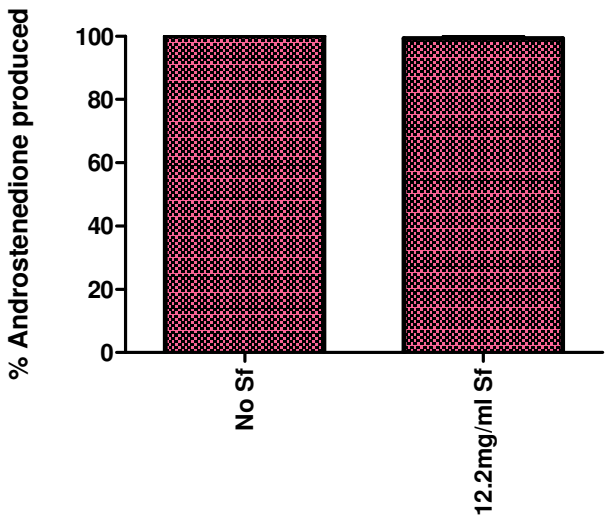


Figure 5.17: Conversion of testosterone to androstenedione in untransfected COS1 cells to determine the effect of 12.2 mg/ml methanol *S. frutescens* extract. Initial [testosterone], 1µM: Sf, *S. frutescens*. Results are representative of one experiment with triplicate data points. Error bars represent SEM.

The maximum concentration of methanol extract of *S. frutescens* assayed was 12.2 mg/ml and no significant inhibition of androstenedione production ($P>0.05$) was observed at this concentration.

5.3.5 Influence of the solvent extracts of the methanol *S. frutescens* extract on substrate conversion

The influence of petroleum ether, ethyl acetate, butanol and residual aqueous extracts on substrate conversion by baboon CYP11B1 was investigated in COS1 cells. The extracts, were added to the COS1 cells in a culture medium containing 1 µM DOC. Since the dried extracts had been prepared five times more concentrated than the methanol extract of *S. frutescens,* the influence of the extracts were assayed using 10 µl while 50 µl of methanol extract of *S. frutescens* was assayed. These volumes were equivalent to 0.58 mg/ml petroleum ether, 0.52 mg/ml ethyl acetate, 0.25 mg/ml butanol, 0.19 mg/ml residual and 2.66 mg/ml methanol extract of *S. frutescens.*

The extracts show significant inhibition of the conversion of DOC to corticosterone with their inhibitory effects being similar ($P<0.05$), with the exception of the ethyl acetate

extract (Figure 5.18). The strongest inhibitory effect was elicited by the petroleum ether extract and was comparable to the methanol extract of *S. frutescens*, inhibiting the formation of corticosterone by approximately 50 % ($P<0.05$). Although the ethyl acetate extract does elicit a significant inhibitory effect ($P<0.05$), it is markedly less that the effect elicited by the petroleum ether extract even though the yield of both extractions were similar (58 mg and 52 mg, respectively). The yield of the butanol phase and residual aqueous extract was approximately half of that of the other two phases (25 mg and 18 mg, respectively), the inhibitory effect elicited by these extracts was 60 % and was not significantly different from the inhibitory effect elicited by the petroleum ether ($P<0.05$). It is therefore possible that these two extracts could contain more potent inhibitory compounds than the petroleum ether extract. The concentration could also influence the inhibitory potential of the compounds present in the extracts. Although LC-MS analysis of the extracts does not give any indication of the concentrations of compounds present, it can be seen that hydrophobic compounds are present, as expected, in the petroleum ether and ethyl acetate extracts as well as in the butanol extract, while the more hydrophilic compounds were present in the residual aqueous extract as well as in the butanol extract. As the more hydrophobic compounds are present in the petroleum ether extract and at higher concentrations, one would have expected this extract to exhibit greater interaction with CYP11B1 based on the hydrophobic nature of the active pocket of the enzyme.

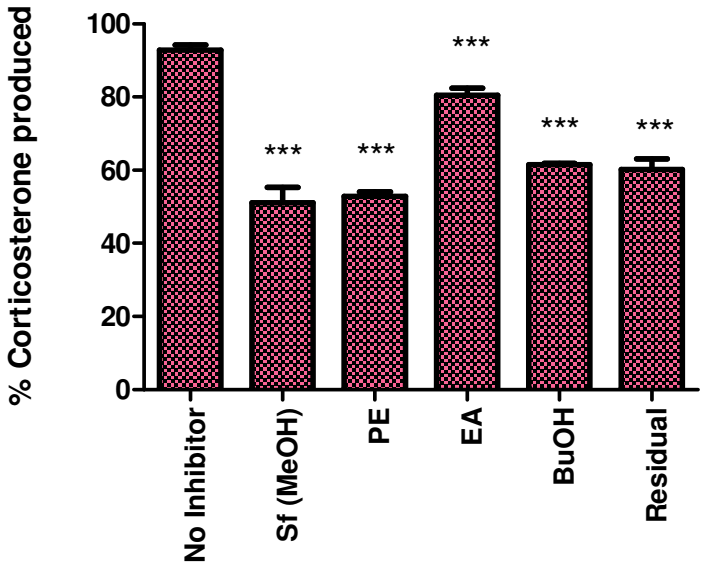


Figure 5.18: Inhibition of the conversion of DOC to corticosterone in COS1 cells expressing baboon CYP11B1 after 8 hours. The initial [DOC] is 1 µM. [Sf (MeOH), methanol *S. frutescens* extract], 2.66 mg/ml; [PE, petroleum ether], 0.58 mg/ml; [EA, ethyl acetate], 0.52 mg/ml; [BuOH, butanol], 0.25 mg/ml and [Residual, residual aqueous extract], 0.19 mg/ml. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$, ***).

Since the extractions had a similar inhibition profile, with the exception of the ethyl acetate extract, and the compounds were not restricted to specific phases without further purification, it was decided to fractionate the methanol extract of *S. frutescens* by HPLC to allow the fractionation in terms of hydrophobicity.

5.3.6 HPLC fractionation of methanol extracts of *S. frutescens*

The sequential extractions of the methanol extract of *S. frutescens* still contained too many complex compounds to be able to specifically attribute the bioactivity of *S. frutescens* to one of the four extractions. The HPLC chromatogram of the methanol extract of *S. frutescens* is shown in Figure 5.19 and the fractions that were collected are indicated as 1-5. These fractions were dried, resuspended in water and their bioactivity was assessed using substrate binding and conversion assays.

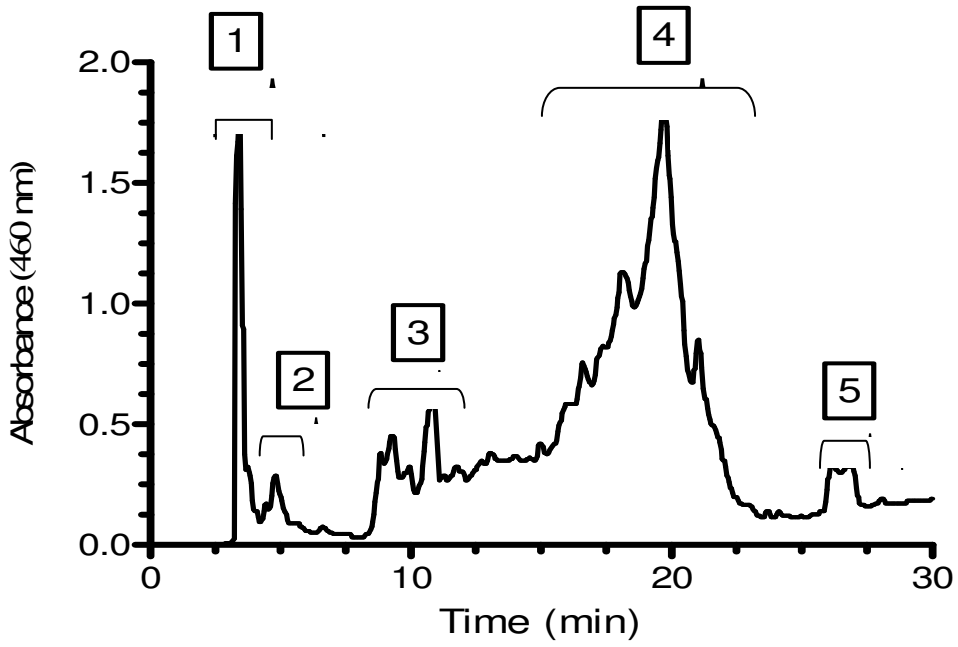


Figure 5.19: Chromatogram of the separation of the methanol extract of *S. frutescens* by HPLC. The fractions that were collected are numbered 1-5. The retention times were: F1, 3.25-4.0 minutes; F2, 4.0-6.0 minutes; F3, 8.30-11.0 minutes; F4, 16.0-22.30 minutes and F5, 25.45-27.30 minutes.

5.3.7 Influence of *S. frutescens* fractions on substrate binding and substrate conversion

The five fractions (F1-5) were isolated as shown in Figure 5.19, dried and resuspended in de-ionized water. The inhibition of the binding of DOC to CYP11B1 in ovine adrenal mitochondria by the fractions is shown in Figure 5.20. The substrate concentration was 8 µM and the cytochrome P450 concentration was 0.88 µM.

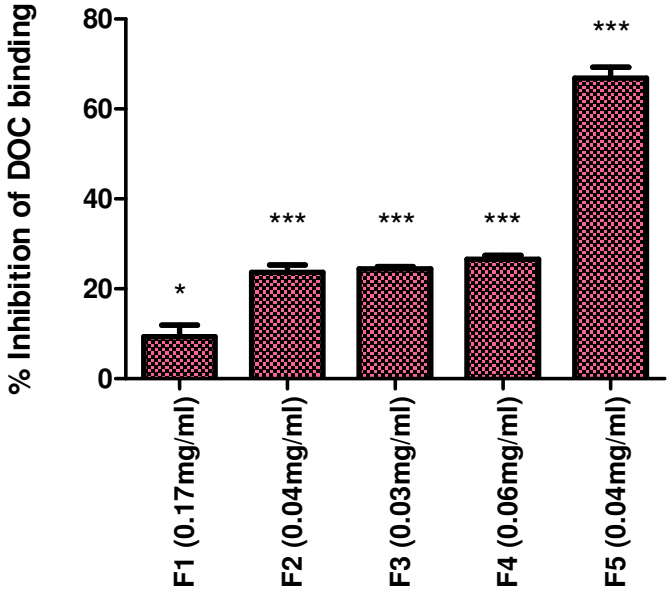


Figure 5.20: Inhibition of DOC (8 μM) binding to CYP11B1 in ovine adrenal mitochondria. [F1], 0.17 mg/ml; [F2], 0.04 mg/ml; [F3], 0.03 mg/ml; [F4], 0.06 mg/ml; [F5], 0.04 mg/ml; [P450], 0.88 μM; [Protein], 0.76 mg protein/mg mitochondrial powder. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$).

Although all the fractions inhibited the binding of DOC to ovine adrenal mitochondrial CYP11B1 significantly ($P<0.05$), fraction 5 elicited the greatest inhibitory effect. Although fraction 1 contained more plant material, the percentage inhibition elicited by this fraction was less than that observed with the other fractions. As fraction 5 shows the highest inhibition, the bioactivity observed in the methanol extract of *S. frutescens* could be attributed mainly to hydrophobic compound(s) present in fraction 5. The data correlates with the inhibition obtained with the petroleum ether extract (Figure 5.18).

The bioactivity of fractions 1-5 was subsequently investigated in COS1 cells expressing baboon CYP11B1. Although the concentrations of the fractions differed from each other, ranging from 0.64 mg/ml to 4.2 mg/ml, these concentrations are representative of the ratios in which the fractions are present in the methanol extract of *S. frutescens* and eluted from the HPLC during fractionation. The only fraction that inhibited the conversion of

DOC to corticosterone by CYP11B1 significantly was fraction 5. Both this fraction and the methanol extract of *S. frutescens* inhibited the conversion of DOC by 100 % (Figure 5.21). This indicates that the bioactive compound(s) present in fraction 5 which inhibited the binding of the substrate to the enzyme, also inhibited the catalytic activity of the enzyme. Compounds in fraction 5 represent the more hydrophobic compounds in *S. frutescens* as they are retained on the C18 column, and elute towards the end of the chromatographic separation.

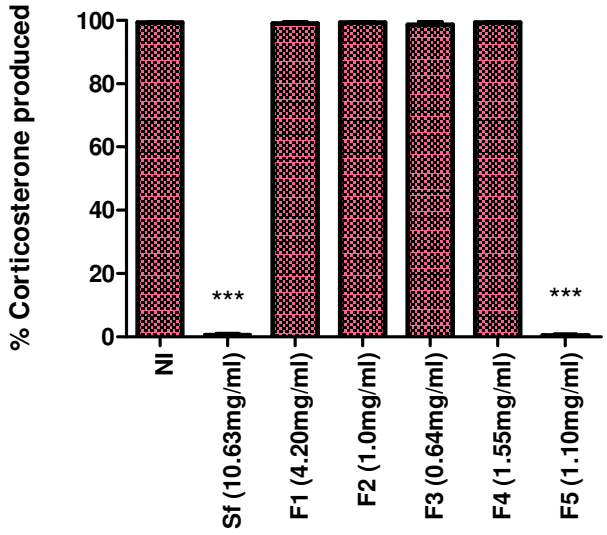


Figure 5.21: Inhibition of the conversion of DOC to corticosterone in COS1 cells expressing baboon CYP11B1 in the presence of the methanol extract of *S. frutescens* (Sf) and fractions 1-5 (F1-F5) after 8 hours. Initial [DOC], 1 µM; [Sf], 10.63 mg/ml; [F1], 4.2 mg/ml; [F2], 1 mg/ml; [F3], 0.64 mg/ml; [F4], 1.55 mg/ml and [F5], 1.1 mg/ml. NI, no inhibitor. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$, ***).

In the previous experiment (Figure 5.18), inhibition of DOC conversion by baboon CYP11B1 was elicited by the butanol extract which contained a mixture of hydrophilic and hydrophobic compounds as seen in Figure 5.2.C. During the fractionation of the methanol extract of *S. frutescens* the hydrophilic compounds would elute from the

column fist, resulting in the presence of the hydrophilic compounds in fractions 1, 2 and 3. These fractions, however, elicited no inhibitory effect contrary to the inhibitory effect elicited by the butanol extract. This may indicate that the hydrophilic compounds may have lost activity during the isolation process. Although fraction 4 possibly consists of a mixture of hydrophobic and hydrophilic compounds, the possibility of a loss of activity of the compounds within this fraction also exists. However, as no bioactivity was observed it may be possible that the compounds in all the fractions are labile and may, upon further purification and exposure to light during the isolation process, have degraded. The fractions were therefore isolated using the same method while protecting the isolated fractions from light whenever possible. A conversion assay was conducted with the latter fractions to determine if the exposure to light had affected the bioactivity of the earlier fractions. In addition it was decided to assay at lower concentrations than those used in the previous experiment. The lower concentrations would allow some comparison of the methanol extract of *S. frutescens* and fraction 5 with the other fractions, if the methanol extract of *S. frutescens* and fraction 5 did not inhibit the catalytic activity completely (as seen in Figure 5.21).

A five-fold decrease in the concentration of the methanol extract of *S. frutescens* resulted in a 50 % inhibition of the conversion of DOC to corticosterone by CYP11B1 in COS1 cells ($P<0.05$). Although there was no significant difference between the percentage inhibition elicited by fraction 4 and 5 at the concentrations assayed ($P>0.05$), the inhibition elicited by fraction 4 and 5 was comparable to the methanol extract of *S. frutescens* (Figure 5.22). The inhibitory effect elicited by these fractions indicates the presence of more stable compound(s) than in the earlier fractions. Although fractions 1-3 elicited an inhibitory effect, the inhibition was still low with fraction 2 inhibiting substrate conversion by 25 % ($P<0.001$). However, the inhibitory activity exhibited by these fractions after having been shielded from light does indicate the presence of labile compounds.

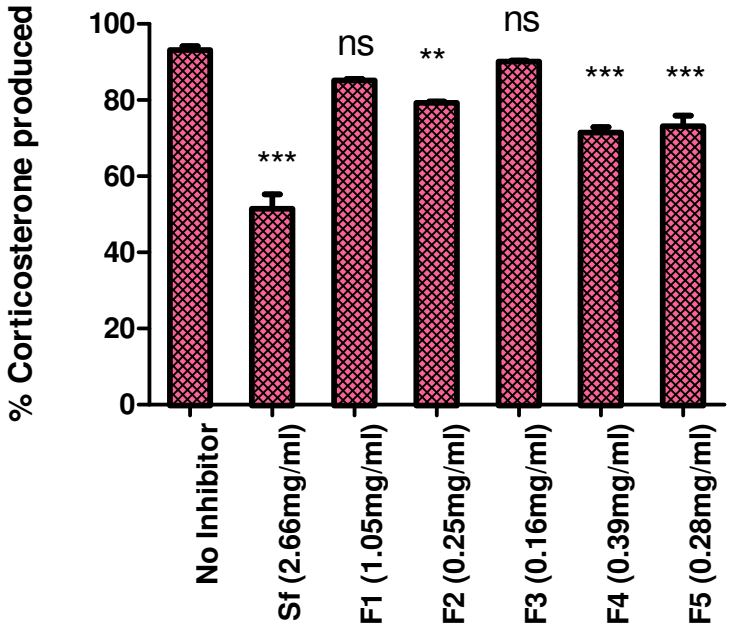


Figure 5.22: Inhibition of the conversion of DOC to corticosterone in COS1cells expressing baboon CYP11B1 in the presence of methanol extract of *S. frutescens* extracts (Sf) and light-protected fractions 1-5 (F1-F5) after 8 hours. The initial [DOC] is 1 µM, [Sf], 2.66 mg/ml; [F1], 1.05 mg/ml, [F2], 0.25 mg/ml, [F3], 0.16 mg/ml; [F4], 0.39 mg/ml and [F5], 0.28 mg/ml. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$).

5.3.8 Influence of flavonoid compounds on substrate binding

LC-MS confirmed the presence of vitexin, rutin and orientin and the bioactivity of these flavonoids were subsequently assayed. The influence of vitexin, rutin and orientin on the binding of DOC to CYP11B1 in ovine adrenal mitochondria was investigated using spectral binding assays and is shown in Figure 5.23. Flavonoids, 10 µM of each, were added to the mitochondrial suspension in the presence of 3.2 µM DOC.

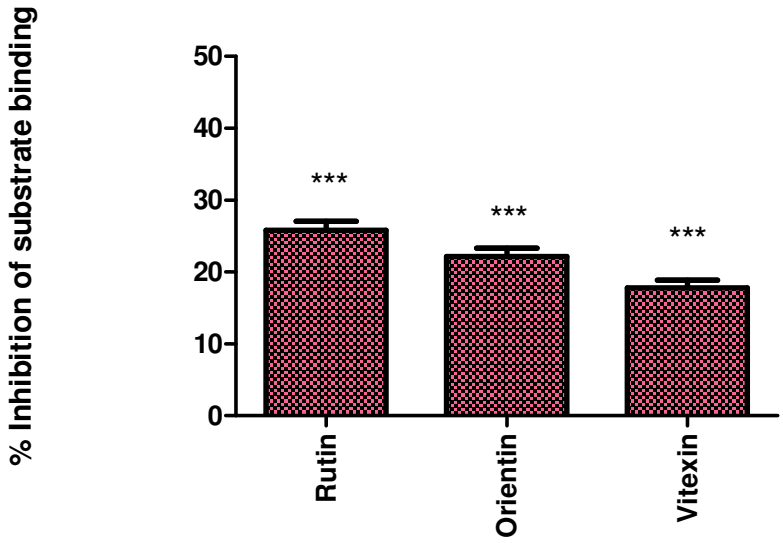


Figure 5.23: Inhibition of DOC binding to CYP11B1 in ovine adrenal mitochondria. [P450], 0.88 µM; [DOC], 3.2 µM; [Rutin], [vitexin] and [orientin], 10 µM; [Protein], 0.76 mg protein/mg mitochondrial powder. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$, ***).

Vitexin, rutin and orientin showed inhibition of substrate binding using spectral binding assays. Rutin had the strongest inhibitory effect on the binding of DOC to CYP11B1, 25 % inhibition ($P<0.05$) (Figure 5.23).

Since the flavonoids were shown to inhibit the binding of the steroid substrate to the CYP11B1, the inhibition was further investigated by assaying binding inhibition in the presence of flavonoid concentrations varying from 10-40 µM (Figure 5.24).

At the lower concentrations rutin remained the stronger inhibitor of DOC binding to CYP11B1, and while vitexin and orientin elicited similar inhibition of DOC binding the three flavonoids did not differ significantly from each other ($P>0.05$). At the higher concentration of 40 µM, rutin and vitexin inhibited DOC binding to a similar degree ($P>0.05$), whereas orientin elicited a lower inhibitory effect ($P<0.05$).

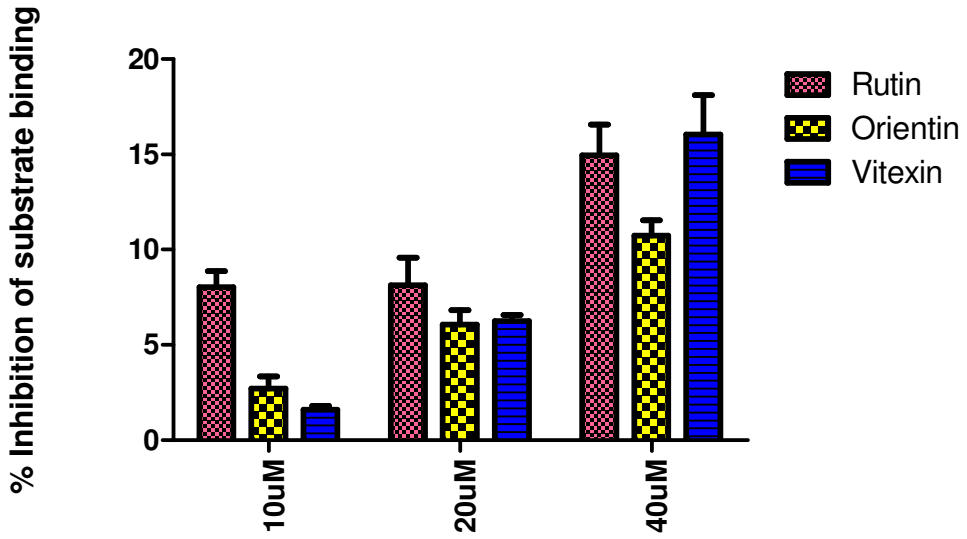


Figure 5.24: Inhibition of DOC binding to CYP11B1 in ovine adrenal mitochondria. [Rutin, orientin and vitexin, respectively], 10-40 µM, [DOC], 3.2 µM, [P450], 0.88 µM, [Protein], 0.76 mg protein/mg mitochondrial powder. Results are representative of three independent experiments. Error bars represent SEM.

The substrate binding data was subsequently plotted generating a double-reciprocal plot to visualize the inhibition data and determine the potency of the individual flavonoids. The data are shown in Figure 5.25.

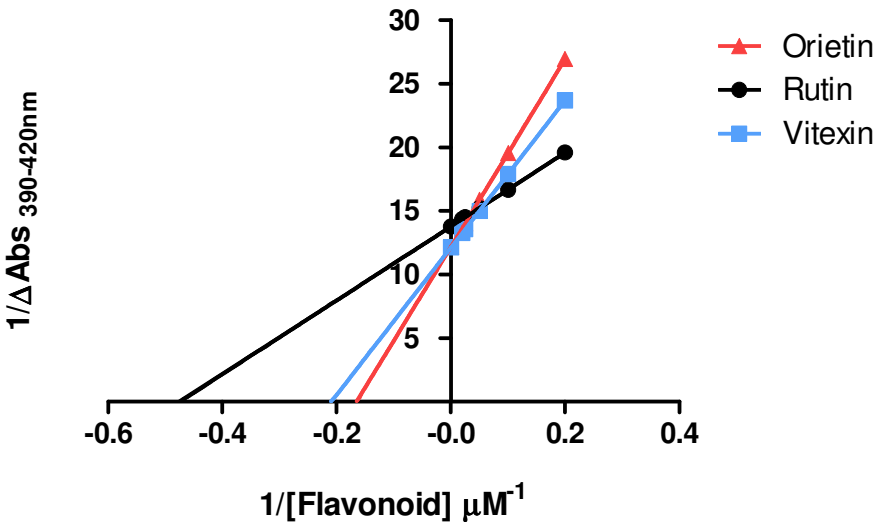


Figure 5.25: Double reciprocal plot of the inhibitory effect of vitexin, orientin and rutin, respectively, on DOC binding in ovine adrenal mitochondria. [P450], 0.88 µM; [DOC], 4 – 32 µM; [vitexin], [orientin] and [rutin], 10, 20, 40 µM, respectively. Results are presented as the mean of three experimental values.

The K_s values for rutin, vitexin and orientin are 2.10 µM, 4.78 µM and 6.06 µM, respectively, implying that 50 % inhibition of the binding of DOC to CYP11B1 is obtained with lower concentrations of rutin. This data confirmed that rutin was a more potent inhibitor of the binding of DOC to CYP11B1 than vitexin and orientin.

From the above results it is clear that the greatest inhibitory effect on substrate binding and substrate conversion by CYP11B1 is still induced by the methanol extract of *S. frutescens*. To determine whether this effect could be attributed to the synergistic action of rutin, vitexin and orientin, the flavonoids were added to the mitochondrial suspension in a ratio of 1:1. The data shown in Figure 5.26 show that the simultaneous addition of two flavonoids, for example 20 µM rutin + 20 µM orientin or 20 µM vitexin + 20 µM orientin, does not inhibit substrate binding to a greater extent than that elicited by addition of the 40 µM of the single flavonoid. There was also no significant difference between the inhibition of DOC binding to CYP11B1 upon the simultaneous addition of 20 µM of each of rutin, vitexin and orientin and the addition of 40 µM of the individual flavonoids ($P>0.05$). A lower percentage inhibition is observed when 20 µM of vitexin,

20 µM orientin and 20 µM rutin are added (1:1:1) simultaneously to the enzyme suspension compared to the presence of 40 µM of the single flavonoid. The greater inhibitory effect elicited by the lower flavonoid concentration (40 µM) possibly indicates that the enzyme is stimulated, rather that inhibited, in the presence of 20 µM of vitexin, 20 µM orientin and 20 µM rutin.

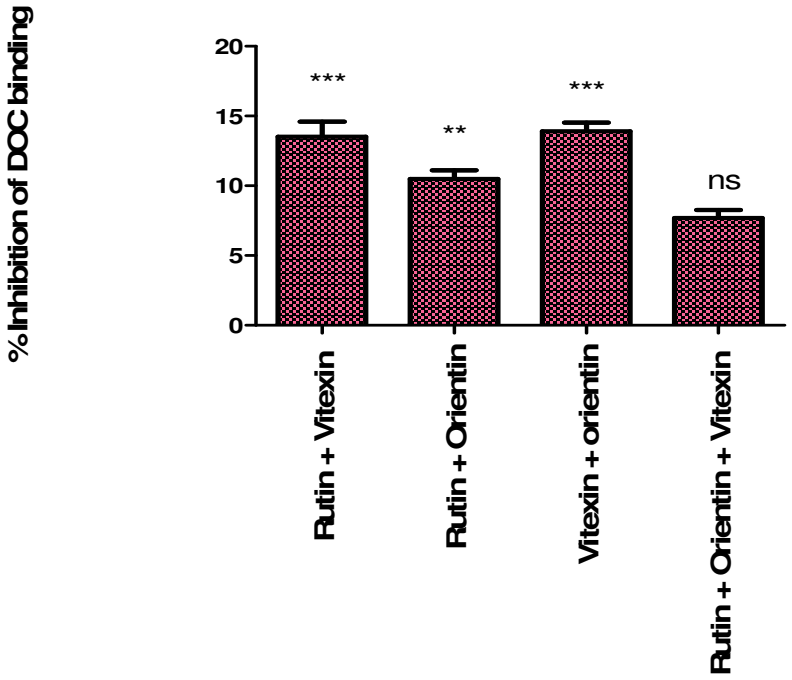


Figure 5.26: Inhibition of DOC binding to ovine adrenal mitochondrial CYP11B1 in the presence of 20 µM rutin, orientin and vitexin, respectively. [P450], 0.88 µM; [Protein], 0.76 mg protein/mg mitochondrial powder; [DOC], 3.2 µM; [Rutin] and [Vitexin], 20 µM of each; [Rutin] and [Orientin], 20 µM of each; [Vitexin] and [Orientin], 20 µM of each; [Rutin], [Orientin] and [Vitexin], 20 µM of each. Results are representative of one experiment with triplicate time points. Error bars represent SEM. The columns were compared using a one-way ANOVA followed by Bonferroni's comparison test ($P<0.05$, ***).

The inhibition of DOC binding to CYP11B1 in ovine adrenal mitochondria was not increased by the presence of different combinations of the flavonoids which suggests that there is no significant synergistic inhibitory action between these specific flavonoids.

5.3.9 The influence of flavonoid compounds on substrate conversion

Orientin, vitexin and rutin were assayed to determine whether these flavonoids could inhibit the catalytic activity of CYP11B1. The conversion of DOC to corticosterone was thus assayed in COS1 cells in the presence of the flavonoids. In Figure 5.27 it is shown that none of the flavonoids had a significant inhibitory effect on the conversion of DOC to corticosterone in COS1 cells (P>0.10).

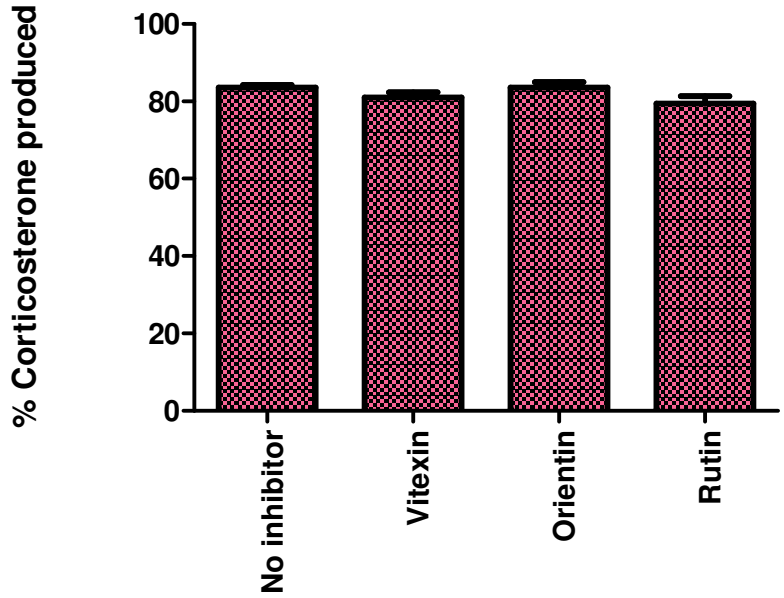


Figure 5.27: Inhibition of the conversion of DOC to corticosterone in COS1 cells expressing baboon CYP11B1 in the presence of 10 µM rutin, orientin and vitexin after 8 hours. The initial [DOC] is 1 µM. Results are representative of three independent experiments. Error bars represent SEM.

Although substrate binding to mitochondrial CYP11B1 was inhibited by 10 µM flavonoids with rutin being the more potent inhibitor, substrate conversion by CYP11B1 in COS1 cells was not inhibited in the presence of vitexin, rutin or orientin at 10 µM.

5.3.10 Influence of SU1 on substrate binding

Although rutin, vitexin and orientin inhibited the binding of DOC by CYP11B1, the effects they elicited were small compared to that of the original methanol extract of *S. frutescens*. This suggests that the bioactivity of *S. frutescens* extracts is possibly attributed to other compounds. Sutherlandioside A (SU1) is a cycloartane glycoside recently identified in *S. frutescens* extracts and there have been several suggestions that this compound may contribute to the medicinal properties of *S. frutescens* extracts, specifically anti-stress [Van Wyk & Wink (2004); Fu *et al* (2008)]. The inhibitory potential of SU1 was investigated by carrying out a spectral binding assay. The binding of DOC to ovine adrenal mitochondrial CYP11B1 was conducted in the presence of 2.4 µM SU1. At this concentration the presence of the SU1 appeared to stimulate the binding of DOC to CYP11B1 (Figure 5.28). DOC binding to CYP11B1 was significantly stimulated by 38.5 % in the presence of SU1 ($P<0.001$). Due to limited availability of the compound, the experiment could unfortunately not be repeated and no further experiments investigating the bioactivity of SU1 could be performed.

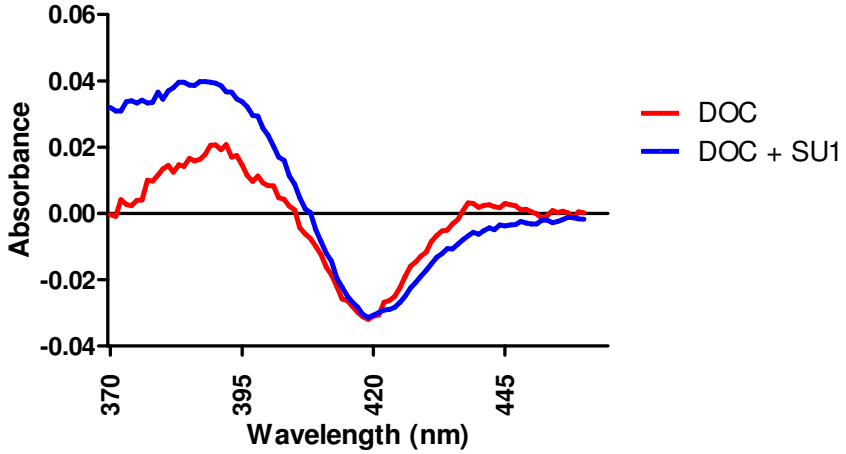


Figure 5.28: Type I substrate-induced difference spectra of DOC binding to ovine adrenal mitochondrial CYP11B1 in the absence and presence of SU1. [DOC], 3.2 µM; [SU1], 2.4 µM, [Protein], 0.76 mg protein/mg mitochondrial powder and [P450], 0.88 µM. Results are from a single experiment.

5.4 Discussion

The methanol extract of *S. frutescens*, fractionations of the extract and compounds identified within these extracts have all been shown to interact with the steroidogenic cytochrome P450 enzyme CYP11B1 by either inhibiting substrate binding or substrate metabolism.

The methanol extract of *S. frutescens* inhibited the binding of both deoxycortisol and DOC to CYP11B1 in ovine adrenal mitochondria. Substrates, at respective concentrations of 8μM and 32 μM, were tested in the presence of 0.21 mg/ml, 0.42 mg/ml and 0.84 mg/ml methanol extract of *S. frutescens*. The respective K_s values (Figure 5.10), 0.13 mg/ml and 0.37 mg/ml, indicated that the binding of deoxycortisol to CYP11B1 was more sensitive to inhibition by the methanol extract of *S. frutescens* than the binding of DOC to CYP11B. A 50 % inhibition of DOC (8 μM) binding (Figure 5.8) was observed in the presence of 0.84 mg/ml extract ($P<0.01$) while 100 % binding inhibition of deoxycortisol (Figure 5.7) was seen at this concentration. In addition, the binding of deoxycortisol and DOC to CYP11B1 in ovine adrenal mitochondria is dose dependent.

In COS1 cells expressing baboon CYP11B1, the conversion of both deoxycortisol and DOC by this enzyme was investigated in the absence and presence of the methanol extract of *S. frutescens* (Figure 5.14 and 5.15). At a concentration of 4.8 mg/ml, the methanol extract of *S. frutescens* extract inhibited the conversion of deoxycortisol to cortisol and DOC to corticosterone by 76 % and 91.5 %, respectively. Since the extracts alone had no effect on cell viability, the inhibition of substrate conversion by CYP11B1 can be attributed to the effect of the methanol extract of *S. frutescens* on the catalytic activity of the enzyme and not to cell death. Also, this experiment suggests that the conversion of DOC is more sensitive to inhibition by the methanol extract of *S. frutescens* than the conversion of deoxycortisol.

The above experiments confirmed that the methanol extract of *S. frutescens* influenced the activity of CYP11B1. It is probable that the observed bioactivity of this extract is due to the presence of a complex mixture of compounds in the extract. In an attempt to identify and isolate some of these compounds, the methanol extract of *S. frutescens* was subjected to further sequential extraction by petroleum ether, ethyl acetate and butanol

(Figure 5.18). The residual aqueous extract was also dried and assayed. The bioactivity of these extractions was tested in COS1 cells expressing baboon CYP11B1 and it was shown that they all elicited an inhibitory effect on the conversion of DOC, with the petroleum ether extraction exhibiting significant inhibition ($P<0.05$). Although the petroleum ether elicited the greatest inhibitory effect, the concentration of this extract (52 ng/ml) used in the conversion assay was almost two fold that of the butanol and residual aqueous extract (25.2 ng/ml and 18.6 ng/ml, respectively). The butanol and residual aqueous extracts also inhibited the conversion of DOC significantly ($P<0.05$). This may imply that the butanol and residual aqueous extracts may contain compounds that are more potent inhibitors of CYP11B1 than the compounds present in the petroleum ether extracts.

In an attempt to further isolate compounds from the methanol extract of *S. frutescens*, this extract was subjected to HPLC fractionation, and five fractions were collected. The bioactivity of the fractions was also assessed by investigating the effect of these fractions on DOC binding and conversion by CYP11B1. The ability of DOC to bind to CYP11B1 in ovine adrenal mitochondria was assayed and although all the fractions inhibited DOC binding ($P<0.05$), fraction 5 elicited the greatest inhibitory effect (Figure 5.20). The inhibition of binding elicited by fraction 5 was significantly different ($P<0.05$) from the inhibition elicited by fractions 1-4. Fraction 5 inhibited the binding of DOC to CYP11B1 by 66 % ($P<0.05$). Although the yield of dried material of fractions 1 is approximately three fold more than the yield of dried material of fractions 2-5, the lowest percentage inhibition of binding was elicited by fraction 1indicating possible degradation of the more labile hydrophilic compounds. The conversion of DOC to corticosterone in COS1 cells expressing baboon CYP11B1 was also inhibited (Figure 5.21). In the presence of the fractions, it was fraction 5 only that inhibited DOC conversion (100 %). Since inhibition was seen with the butanol extract which contained hydrophilic compounds, it would have been expected that the fractions containing the hydrophilic compounds, fraction 1, 2 and 3, would also elicit an inhibitory effect on the conversion of DOC by CYP11B1. These fractions, however, elicited no inhibitory effect even though the butanol extract,

containing hydrophilic compounds, elicited an inhibitory effect Thus, the hydrophilic compounds present in fractions 1-3 may have lost activity during the isolation process.

Light is known to degrade secondary metabolites and labile plant products. For this reason the effect of light during the fractionation was investigated. All the samples protected from light inhibited the conversion of DOC significantly with fraction 4 and 5 exhibiting the strongest inhibition (Figure 5.22). Although there was no significant difference in the inhibition elicited by fraction 4 and 5 ($P>0.05$), the inhibitory effect of fraction 4 and 5 did significantly differ from the effect elicited by fraction 1, 2 and 3 ($P<0.05$). The LC-MS analysis of the fractions confirmed a marked difference in the compounds present in the light-exposed and the light-protected fractions. Although the majority of the compounds in the fractions appeared to be degraded upon exposure to light, the compounds present in fraction 5 did not. These results suggest that fraction 4 may contain bioactive compounds that are labile, while the compounds in fraction 5 are relatively stable. The fact that fractions 4 and 5 elicit greater inhibition of substrate binding and conversion than the other fractions may be attributed to the hydrophobicity of the compounds in these fractions. Fractions 4 and 5 elute later from the column, being more hydrophobic than fractions 1-3. Considering the structures of the steroid substrates of CYP11B1 (Figure 4.10 and 4.11), hydrophobic compounds may be able to interact with this enzyme to a greater extent than hydrophilic compounds. On the other hand, the butanol extracts, which yielded approximately half of the dried extract weight of the petroleum ether and ethyl acetate extracts, elicited an inhibitory effect similar to that of the petroleum ether extract. This may imply that the hydrophilic compounds also elicit an inhibitory effect and may be more potent inhibitors than the hydrophobic compounds. In addition, it is possible that the compounds within the fractions work synergistically to elicit the inhibitory effect.

In an attempt to identify compounds within fractions 1-5 and the petroleum ether, ethyl acetate, butanol and residual aqueous extracts, these were analyzed by LC-MS. Five flavonoid standards, which have been identified in other medicinal plants, and SU1 were available for analysis. Rutin, orientin and vitexin were identified in the fractions and in the petroleum ether, ethyl acetate, butanol and residual aqueous extracts. Rutin was found

in the petroleum ether, ethyl acetate, butanol and residual aqueous extract and fraction 4 (Table 5.3). Orientin was found in fraction 1 and 4 and in the petroleum ether and residual aqueous extracts, with the highest levels present in the fraction 1. Vitexin was found in fraction 2 and in the residual aqueous extracts. Iso-vitexin and iso-orientin were not identified in any of the extracts or fractions. SU1 was identified in the petroleum ether, ethyl acetate, butanol and residual aqueous extracts as well as fraction 4 and 5. Of all the tested compounds, SU1 appeared to be the most stable upon light exposure. There is a marked difference in the flavonoid content of the light-exposed and light-protected fractions. The exposure to light may have degraded the flavonoids during the fractionation process, thus influencing the bioactivity of the fractions.

S. frutescens extracts contain complex mixtures of compounds and the presence of the flavonoids in a particular fraction or extract may not be solely responsible for the inhibitory effects elicited by the fraction or extract. To determine whether the flavonoids could elicit an inhibitory effect on CYP11B1, they were firstly investigated individually (Figure 5.23 and 5.24) and subsequently combined in assays to investigate the possibility of synergistic action (Figure 5.26).

Although rutin, orientin and vitexin (10 µM) did not inhibit the conversion of DOC to corticosterone significantly, these flavonoids did inhibit DOC binding to CYP11B1 in adrenal mitochondria. At lower concentrations (10 µM), rutin elicited the greatest inhibitory effect on DOC binding to CYP11B1. At higher concentrations (40 µM) vitexin and rutin inhibited DOC binding to a similar degree (~15 %). In addition, when the K_s values of the flavonoids were compared (Figure 5.25), it was confirmed that rutin was the more potent inhibitor with a K_s of 2.10 µM. Although rutin, orientin and vitexin inhibited the binding of DOC, the inhibition elicited by these flavonoids was significantly ($P<0.05$) less than the inhibition observed in the presence of the methanol extract of *S. frutescens* and fractions, indicating that these flavonoids are not the only bioactive compounds in the extracts.

The possibility of synergistic action between rutin, orientin and vitexin was investigated by assessing the effect that combinations of these flavonoids may have on the binding of DOC to CYP11B1. The data shows that these flavonoids do not synergistically affect

substrate binding, as the observed inhibitory effect was similar to the inhibition elicited by the individual flavonoids. Interestingly, this is not the case when all three flavonoids are added simultaneously (7.7 % inhibition). This is significantly less ($P<0.10$) than the individual flavonoids and might be attributed to a synergistic adaptogenic effect.

SU1 is a cycloartane glycoside that was recently isolated from *S. frutescens*. The presence of SU1 was confirmed in the methanol extract of *S. frutescens,* as well as the petroleum ether, ethyl acetate, butanol, residual aqueous extracts and fractions with the highest levels present in the ethyl acetate and butanol extracts. The bioactivity of this compound was investigated in spectral binding assays which showed that this compound appeared to stimulate DOC binding to CYP11B1. The state of the heme iron of P450 enzymes changes upon substrate binding from a low to a high spin state. The change in spin state results in a change in the spectral properties of the enzyme with an increase in absorbance at 390 nm and a decrease in absorbance at 420 nm. A significant increase ($P<0.001$) was seen in the amplitude of the curve (Figure 5.28). It is thus possible that SU1 may bind to the enzyme and elicit a difference spectrum. In addition, CYP11A1 is also present in the mitochondrial preparation and thus SU1 may bind to CYP11A1 and elicit a spectrum. The stimulation of substrate binding by SU1 may enhance the activity of the enzyme, possibly offering an explanation for the adaptogenic activity that was observed by Smith and Myburgh in non stressed rats receiving *S. frutescens*. The non-stressed rats receiving *S. frutescens* exhibited significantly increased plasma corticosterone levels compared to the non-stressed rats not receiving *S. frutescens*. *S. frutescens* thus acts as an adaptogen in the non-stressed rats [Smith & Myburgh (2004)].

In summary, the extracts the fractions of *S. frutescens* and influenced the binding to and the conversion of steroid substrates by CYP11B1. The bioactivity observed could not be attributed to the identified flavonoids alone. In addition, the bioactivity of the flavonoid compounds was assayed at very low concentrations and it may be possible that the flavonoids elicit bioactivity at concentrations greater than 40 μM. The inhibitory effect elicited by the methanol extract of *S. frutescens* was significantly greater than that of the flavonoids, the solvent extractions and the fractions, indicating that other unidentified compounds may be contributing to the inhibitory effects of *S. frutescens*. Although rutin,

orientin and vitexin displayed no synergistic action, an inhibitory effect may be elicited through the synergistic action of unknown compounds. The ability of these extracts and compounds to interfere with the normal function of CYP11B1, ultimately influencing glucocorticoid production, could partially explain the use of *S. frutescens* to treat the symptoms of chronic stress.

Chapter 6

Conclusion

Southern Africa boasts a rich floral diversity which has been exploited in traditional medicinal practices. In spite of westernization and urbanization, approximately 27 million South Africans still rely on traditional medicines for their primary healthcare. Considering that many mainstream medicines are of plant origin, such as digitalis, chinchona and opium poppy, there may be merit in the use of medicinal plants.

Since most plant extracts contain complex mixtures of compounds, the effect of traditional medicine may be elicited by the ability of these compounds to influence several different targets. In doing so, a greater physiological response may be elicited with perhaps minimal side effects. In contrast, pharmaceuticals, however, usually consist of a single chemical entity designed for a single target, usually producing side-effects. The ability of natural products to interact with various physiological targets simultaneously could lead to cross-reactions with pharmaceuticals and caution should be taken when natural products are taken in conjunction with pharmaceuticals.

S. frutescens is traditionally used to treat a variety of diseases including cancer, diabetes, stress and anxiety, wounds, pain relief, fever, infections, tuberculosis, peptic ulcers and wasting in HIV and cancer patients [Van Wyk *et al* (2000); Van Wyk *et al* (2008); Van Wyk & Albrecht (2008)]. The therapeutic effects associated with *S. frutescens* have been attributed to a mixture of complex compounds present in these extracts, including GABA, pinitol, canavanine and triterpenoids. It has been suggested that the ability of different compounds within plant extracts to simultaneously bind to different targets may be responsible for the bioactivity of such plant extracts. Therefore, the same principle may apply to *S. frutescens* extracts: the presence of complex compounds within the *S. frutescens* extracts may be responsible for its bioactivity. Although plausible, the synergistic action may not be attributed to the known compounds in *S. frutescens*, but rather unidentified compounds. In addition, not only is the identification of other bioactive compounds within *S. frutescens* a topic for further study, but also investigations

into the structural composition of these compounds since structural analysis has also received very little attention.

Scientific investigations, including a clinical trial, have confirmed that the consumption of 800 mg of dried *S. frutescens* leaf powder is safe for adults. Although canavanine is known to induce auto immune diseases in prone individuals, canavanine in *S. frutescens* was metabolized after consumption with no traces of the compound found in the plasma of the subjects in the clinical trial [Johnson *et al* (2007)]. The effects of *S. frutescens* have, to date, not been determined in small children and pregnant or lactating women. Although the clinical trial provides valuable information regarding the safe use of *S. frutescens*, the effects of its long-term use have not yet been determined. The presence of hydrophobic compounds within *S. frutescens* may be noteworthy since these compounds are stored in adipose tissues. The continual use of these plant products over extended periods may thus lead to the accumulation of compounds within the body, possibly reaching toxic levels.

Although the mechanisms through which *S. frutescens* elicits its medicinal effects are relatively unknown, several physiological targets may partly explain the medicinal effects associated with the plant extracts. The inhibition of COX-2 expression by *S. frutescens* may warrant its use as a natural therapy for inflammation. The inhibition of COX-2 is associated with the therapeutic effects of anti-inflammatory drugs and the severe side effects elicited by these drugs are associated with the inhibition of the expression of COX-1. Therefore, by inhibiting the expression of COX-2 selectively, *S. frutescens* may induce the therapeutic effects of an anti-inflammatory drug without eliciting the side-effects.

The inhibition of the expression of COX-2 by *S. frutescens* may also warrant its application in the prevention of tumor formation. The over expression of COX-2 has been linked to an increased susceptibility to tumor formation and COX-2 inhibitors have been shown to prevent cancer [Kakizoe (2003)]. The inhibition of COX-2 expression may be attributed to the ability of *S. frutescens* to suppress the binding of DNA to NF-κB and in so doing prevent the formation of tumors. In addition, the recent identification of the sutherlandiosides [Fu *et al* (2008)] may also contribute to the anti-cancer properties of *S.*

frutescens since cycloartane compounds with similar structures to that of the identified sutherlandiosides are potent inhibitors of mouse skin carcinogenesis.

The anti-oxidant potential of *S. frutescens* may contribute significantly to its application as an anti-inflammatory treatment. In the case of auto-immune diabetes, inflammation causes an increased production of $NO^{\bullet}$ through cytokine activation. The accumulation of $NO^{\bullet}$ causes cell damage and thus impairs insulin production by the β-cells of the pancreas. *S. frutescens* extracts are known to inhibit $NO^{\bullet}$ production thus alleviating the cell damage caused and prevents further damage to the cells. In the case of type 2 diabetes, the insufficient production of insulin stimulates oxidative stress markers. The anti-oxidant potential of *S. frutescens* may attenuate the damage caused by oxidative stress. Thus, the anti-inflammatory and anti-oxidant potential of *S. frutescens* are closely linked by the ability of the compounds within the plant extracts to reduce ROR production. The presence of canavanine in *S. frutescens* may contribute to the anti-oxidant and anti-inflammatory potential of this plant by interfering with arginine uptake which is essential for $NO^{\bullet}$ production.

S. frutescens is a renowned traditional remedy for the treatment of stress and depression. It has been demonstrated that the elevated levels of glucocorticoids that are associated with chronic stress were significantly reduced in stressed rats receiving *S. frutescens* [Smith & Myburgh (2004)]. The decreased plasma glucocorticoid levels may be partly explained through an inhibitory affect that *S. frutescens* has on the P450 enzymes. Prevoo showed that CYP17 and CYP21 are significantly influenced by the presence of *S. frutescens* extracts and may interfere with the production of glucocorticoid precursors, thus resulting in reduced glucocorticoid production [Prevoo (2005)]. This reduction in plasma glucocorticoid levels would alleviate the symptoms associated with chronic stress. Interestingly, *S. frutescens* does not seem to only have an inhibitory effect on glucocorticoid production. When non-stressed rats were fed *S. frutescens*, elevated plasma glucocorticoids were observed indicating a possible adaptogenic effect [Smith & Myburgh (2004)]. It has been suggested that this effect may be elicited through interaction with the P450 enzymes.

A study has confirmed that sutherlandioside B elicits an inhibitory effect on CYP3A4, although no interaction was exhibited with CYP2D6 [Madgula *et al* (2008)]. This documented interaction between a sutherlandioside and a P450 enzyme may imply that these compounds can affect other P450 enzymes since these enzymes are highly homologous. The fact that sutherlandioside B was not metabolized by liver microsomes may have implications for its interaction with the steroidogenic P450 enzymes. If the compound is not metabolized by the liver P450 enzymes it may interact with these enzymes, possibly including interaction with CYP17 and CYP21. This was confirmed by Prevoo *et al* (2005) by demonstrating that the sutherlandioside SU1 was able to inhibit the binding of natural steroid substrates to CYP17 and CYP21 in ovine adrenal microsomes.In addition, it was found that this compound induces a type II difference spectrum, indicating that it binds directly to the heme iron of the P450 enzyme.

The decrease in plasma glucocorticoid levels elicited by *S. frutescens* may also be of significance in alleviating the symptoms of type 2 diabetes. Type 2 diabetes develops due to insulin resistance with long-term exposure to elevated cortisol levels being one of the causative factors. The most common explanation for elevated cortisol levels in type 2 diabetes is the stimulation of glucocorticoid production due to high plasma free fatty acid concentrations, usually as a direct result of a high calorie diet. High calorie diets have also been associated with visceral obesity, indicating a link between visceral obesity and type 2 diabetes. Since the glucocorticoids play a major role in regulating blood glucose, the potential of *S. frutescens* in the treatment of type 2 diabetes may be attributed to the influence of the extracts on glucocorticoid production. *S. frutescens* may influence the steroidogenic P450 enzymes, which are responsible for the production of glucocorticoids and glucocorticoid precursors, or may influence 11β-HSD type I which is over-expressed in adipose tissue and is responsible for the activation of cortisol. This isoform is also expressed in peripheral tissues and contributes indirectly to the regulation of cortisol. The interaction of *S. frutescens* with dehydrogenase enzymes is not well documented and may require further investigation to determine if these enzymes are affected by *S. frutescens*.

It appears that the ailments that are traditionally treated with *S. frutescens* are closely linked to long-term exposure to elevated cortisol levels. Thus by targeting CYP11B1,

which is responsible for cortisol production, the symptoms associated with elevated cortisol levels may be alleviated. This study shows that CYP11B1 is a plausible target for the bioactivity of *S. frutescens* since extracts and fractionations made from the plant, as well as compounds found within the plant extracts, elicited significant inhibitory effects on the binding and catalytic activity of CYP11B1. Although rutin, vitexin and orientin were identified within the plant extracts, these compounds elicited negligible inhibitory effects compared to the methanol extract of *S. frutescens*, indicating the presence of unidentified inhibitory compounds within the extracts. Interestingly, the K_s value of the methanol extract of *S. frutescens* in the presence of deoxycortisol confirmed that the binding of this substrate to CYP11B1 is more sensitive to the presence of the extract than the binding of DOC to the enzyme. However, the catalytic activity of CYP11B1 for the conversion of DOC appeared to more sensitive to the methanol extract of *S. frutescens* than that for deoxycortisol. Due to the complex nature of P450 enzymes, inhibition of these enzymes may occur through various mechanisms including the interaction of the inhibitory compounds with the active site of the enzyme and the interaction of the inhibitory compound with other binding sites on the enzyme influencing the binding of electron carriers. Although it was confirmed that the methanol extract of *S. frutescens* influenced the binding and catalytic activity of CYP11B1, it was not determined which of the abovementioned mechanisms were affected and would require further investigation.

The petroleum ether, ethyl acetate, butanol and residual aqueous extracts inhibited conversion by CYP11B1 significantly. Although rutin is present in the petroleum ether extract, this does not explain the inhibitory effect elicited by this extract, since rutin did not elicit significant inhibitory effects on substrate conversion by CYP11B1. Thus, the effect elicited by the petroleum ether extract may be attributed to the presence of another compound(s) in the extract. Although the ethyl acetate extract contained significant concentrations of rutin and SU1, this extract elicited a significantly lower inhibition of DOC conversion. This may be due to the presence of SU1 which was shown to stimulate substrate binding to CYP11B1, although no conversion assays have been performed to confirm the effect of SU1 on substrate conversion. The butanol extract contained approximately the same concentration of SU1 as the ethyl acetate extract, but

approximately 2 fold more rutin. Although the yield of the butanol extract was only half of the petroleum ether and ethyl acetate extract, the butanol extract elicited an inhibitory effect comparable to that elicited by both the petroleum ether and ethyl acetate extracts. This may indicate that in spite of the stimulating effect SU1 may have on DOC binding to CYP11B1, the butanol extract may contain other compounds that are more potent inhibitors of the catalytic activity of CYP11B1 than the compounds present in the petroleum ether and ethyl acetate extracts. Although the residual aqueous extract appears to contain significant amounts of rutin, vitexin and orientin, the presence of the flavonoids does not account for the inhibitory effect on substrate conversion elicited by this extract. In addition, the residual aqueous extract also yielded approximately half of the dried extract material of the petroleum ether and ethyl acetate extracts. This may imply that, like the butanol extract, the residual aqueous extract may contain more potent inhibitors of CYP11B1 than the petroleum ether and ethyl acetate extracts. LC-MS analysis of the residual aqueous extract revealed a peak caused by a hydrophilic compound with a molecular mass of 361, indicating that a hydrophilic compound possibly contributes towards the bioactivity of this extract. Although this compound is significantly smaller than the flavonoids and SU1 that have been identified in the residual aqueous extract it has a mass similar to that of the steroid substrates of the P450 enzymes. Although further investigations into the characteristics of this compound are required, the LC-MS analysis may possibly indicate a novel compound with the ability to inhibit CYP11B1. Taken together, the findings indicate that the hydrophilic compounds present in these extracts may be more potent inhibitors than the hydrophobic compounds. This indicates that the hydrophilic compounds may elicit an inhibitory effect by binding to the hydrophilic regions of the enzyme binding site.

As expected, the LC-MS analysis and the bioactivity assays confirmed that the exposure to light affected the bioactivity of the five fractions which were isolated using HPLC. Although the yield of the fractions remained the same for both light-exposed and light-protected experiments, the compounds within the fractions differed significantly. The chromatograms of the light-exposed and light-protected fractions showed that several peaks were absent after fractions 1-3 were exposed to light. The loss of bioactive

compounds in fractions 1-3 was confirmed when the light-protected fractions were assayed for activity. Light-protected fractions 1-3 elicited an inhibitory effect whereas no inhibitory activity was exhibited by the corresponding light-exposed fractions. The activity elicited by fractions 1-3 confirms the presence of labile, hydrophilic compounds within *S. frutescens* that elicit an inhibitory effect. Although fraction 2 contains vitexin, the presence of this compound may partially explain the inhibition of substrate binding elicited by fraction 2, but not the inhibition of substrate conversion. No significant difference in inhibition of substrate conversion was elicited by fraction 1, although the light-protected fraction contained a significant amount of orientin. The bioactivity and flavonoid content of fraction 4 was significantly affected by the exposure to light. Although the light-protected fraction 4 exhibited higher concentrations of orientin and rutin, the concentration of vitexin was higher in the light-exposed fraction 4.

Fraction 5 elicited a similar inhibitory effect on CYP11B1 activity irrespective of being exposed to light. Thus, it would appear that the more hydrophobic compounds responsible for the bioactivity in fraction 5 are relatively stable. The only test compound that remained present in the same concentrations upon light exposure is SU1. However, the inhibition of substrate binding cannot be attributed to the presence of this compound, thus indicating the presence of other stable compounds within fraction 5.

According to the yield of dried fraction, the most potent inhibitory compounds are present in fraction 5 and although fractions 2 and 3 elicited a small inhibitory effect, the yield of these fractions was significantly less than that of fraction 4. The data implies that fraction 5 contains the more potent inhibitory compounds, but no conclusive deduction can be made regarding the potency of the different fractions based on yield. Further investigations are thus required to address this.

The identification of rutin, vitexin and orientin in the extracts and fractions partly explained the inhibition of substrate binding to CYP11B1 elicited by the extracts and the fractions. Rutin was the more potent inhibitor of the three flavonoids with 2.10 μM rutin required to inhibit the binding of DOC to CYP11B1 by 50 %. Rutin has more hydroxyl groups attached to its aromatic ring structure than vitexin or orientin which may contribute to its more potent inhibitory effects. It was shown that the inhibitory potency

of 17β-hydroxysteroid dehydrogenase type 5 by flavonoid compounds is linked to the degree of hydroxylation [Krazeisen *et al* (2001)]. However, the flavonoids did not contribute to the inhibitory effect of the extracts and the fractions on substrate conversion by CYP11B1 at the concentrations tested. Further investigations are thus required to determine whether higher concentrations of flavonoids will effect substrate conversion by CYP11B1.

From the results it appears that SU1 stimulates substrate binding to CYP11B1 as a clear increase in the difference in absorption of enzyme-substrate complex, indicated by the peak at 390 nm and trough at 420 nm. The elevated glucocorticoid levels observed in non-stressed rats receiving *S. frutescens* may be explained by the presence of SU1 and other sutherlandiosides in *S. frutescens* and the ability to stimulate binding to CYP11B1. It has previously been documented that compounds that are not natural substrates of P450 enzymes can bind directly to the heme iron of the enzymes and induce type II difference spectra [Prevoo, MSc thesis, 2004]. These compounds are usually isozyme specific due to the compound binding in the enzyme active pocket. This implies that SU1 may have a similar stimulating effect on CYP11B2 and CYP11A1, since these enzymes have substrate binding regions homologous to CYP11B1. Stimulation of CYP11A1 may result in elevated glucocorticoid levels since this is the rate limiting step in steroidogenesis, but stimulation of CYP11B2 may increase aldosterone production rather than glucocorticoid production. The stimulation of CYP11B2 could, however, not be responsible for the adaptogenic effect observed in the non-stressed rats since the major stress hormone in the rat is corticosterone. This implies that CYP11B2 would be inhibited rather than stimulated to allow corticosterone production by CYP11B1. Thus, the possible adaptogenic effect elicited by SU1 might not only be due to its effect on CYP11B1, but also CYP11A1 and CYP11B2. In addition, the increase and decrease in plasma glucocorticoid levels in the stressed and non-stressed rats may also be attributed to an unidentified compound and not solely SU1. There is also the possibility of synergistic actions between SU1 and other compounds within *S. frutescens* which requires further investigation.

In an attempt to identify a compound that may be responsible for the bioactivity of the extracts and fractions regarding substrate conversion by CYP11B1, the LC-MS chromatograms were further analyzed. Although the chromatograms were scanned for the presence of sutherlandiosides C and D, these compounds could not be identified without the presence of a standard to compare retention times and UV spectra. SU1 and sutherlandioside B have the same molecular mass, and could therefore not be distinguished from each other by this method without a standard of both sutherlandiosides to compare UV spectra and retention times. Thus, the identification and isolation of sutherlandiosides B, C and D requires further investigation.

In summary, the effects of the *S. frutescens* extracts and fractions on CYP11B1 provides preliminary scientific appraisal for the use of *S. frutescens* to treat the symptoms of stress, inflammation and diabetes. The inhibition of CYP11B1 is thus a plausible mechanism through which compounds within *S. frutescens* may reduce cortisol production, thus inhibiting the stress and inflammatory responses and inducing glycogen uptake, thereby antagonizing insulin resistance. The stimulatory effect of SU1 on substrate binding to CYP11B1 may contribute to the adaptogenic effect previously observed in non-stressed rats. The contrasting effects of the compounds within *S. frutescens* on CYP11B1 may indicate that although these compounds do interact with CYP17, CYP21 and CYP11B1, the overall medicinal effect of this plant may be due to the simultaneous interaction of compounds with various physiological targets, eliciting a greater physiological response with minimal side effects. This implies that the use of *S. frutescens* products as natural therapies for stress, inflammation and type 2 diabetes may hold advantages over the use of pharmaceuticals.

Chapter 7

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Chapter 8

Abstract

Many plants common to Southern Africa are used as traditional medicines. *S. frutescens* is a traditional medicine used to treat various ailments of which stress, depression and inflammation are the most renowned [Van Wyk & Wink (2004)]. Although preclinical data suggests that the medicinal properties of *S. frutescens* can be attributed to the presence of pinitol, GABA and canavanine, these compounds do not account for all the anecdotal therapies attributed to *S. frutescens*. The complex nature of the compounds present in *S. frutescens* may be responsible for eliciting the medicinal effects associated with this plant by simultaneously targeting several physiological targets.

The selective inhibition of COX-2 and of $NO^{\bullet}$ production are documented physiological targets for the action of *S. frutescens* in the treatment of diabetes, inflammation and stress. Although these physiological targets do provide insight into the mechanisms by which *S. frutescens* may act, another plausible target is the endocrine system, particularly the P450 enzymes. The adrenal P450 enzymes regulate the production of glucocorticoids and inhibition of these enzymes may thus reduce glucocorticoid production. Although *S. frutescens* extracts have been documented to interact with CYP17 and CYP21, it cannot be assumed that these extracts interact with CYP11B1, the enzyme responsible for the conversion of DOC and deoxycortisol to corticosterone and cortisol, respectively. Thus, this study investigates the possible effect that *S. frutescens* extracts have on cortisol production through the inhibition of CYP11B1.

The bioactivity of solvent extractions and fractions of *S. frutescens* was determined through assessing the effect of the extracts and fractions on substrate binding and conversion. The methanol extract of *S. frutescens* elicited the greatest inhibitory effect on substrate binding and substrate conversion. The extracts appear to significantly inhibit the conversion of DOC by CYP11B1, with the highest inhibition elicited by the petroleum ether extract and the lowest by the ethyl acetate extract, suggesting that the more hydrophobic compounds in the petroleum ether extract may be potent inhibitors of CYP11B1. At lower concentrations the butanol and residual aqueous extract elicit an

inhibitory effect similar to that of the petroleum ether extract thus indicating the presence of potent inhibitory compounds within the hydrophilic extracts.

The five isolated fractions showed marked loss of bioactivity upon exposure to light. These fractions showed very little inhibitory effects with the most inhibition elicited by fraction 5. In the case of the fractions which were protected from light, the most inhibition was elicited by fraction 4 and 5. This data indicates the presence of relatively stable hydrophobic compounds in fraction 5.

The extracts and fractions were analyzed for the presence of rutin, vitexin, orientin and SU1. Although the flavonoids inhibited substrate binding to CYP11B1, no synergistic action was observed between the three flavonoids with rutin being the more potent inhibitor of substrate binding. The inhibition of substrate conversion by the flavonoids was negligible at the concentrations assayed. SU1 appeared to stimulate binding to CYP11B1, but no conversion assays could be performed due to limited availability of SU1.

This study shows that CYP11B1 is inhibited by *S. frutescens* indicating a basis for the use of this plant in the treatment of stress, diabetes and inflammation.