An investigation into the stress relieving properties of *Sutherlandia frutescens*: inhibition of steroidogenic cytochrome P450 enzymes

Désirée Prevoo

Thesis presented for the degree Master of Science (Biochemistry) at the University of Stellenbosch

Promoter: Dr. A.C. Swart
Co-promoter: Prof. P. Swart
Department of Biochemistry, University of Stellenbosch
December 2005
Declaration:

I, the undersigned, hereby declare that the work contained in this thesis is of my own original work and has not previously in its entirety or in part been submitted at any university for a degree.
Summary

This study:

I. Investigates the influence of *S. frutescens* on the binding properties of cytochrome P450-dependent enzymes in ovine adrenocortical mitochondria and microsomes, demonstrating that *S. frutescens* extracts elicit difference spectra and inhibit the Type I difference spectra induced by natural steroids.

II. Indicates inhibition by *S. frutescens* extracts of the catalytic activity of cytochrome P450-dependent-17α-hydroxylase and cytochrome P450-dependent-steroid-21-hydroxylase enzymes in ovine adrenocortical microsomes.

III. Describes an assay determining the inhibitory effects of *S. frutescens*, in COS1 cells, on individual cytochrome P450-dependent enzymes — ovine, baboon and human cytochrome P450-dependent-17α-hydroxylase, and bovine cytochrome P450-dependent-21-hydroxylase enzymes.

IV. Demonstrates that the inhibition of elevated plasma glucocorticoid levels in rats exposed to chronic immobilization stress could possibly be attributed to the influence of hydrophilic and hydrophobic compounds in *S. frutescens* on cytochromes P450-dependent enzymes.
Hierdie studie:

I. Ondersoek die invloed van *S. frutescens* op die bindingseienskappe van sitochroom P450-afhanklike ensieme in skaapbynier mitokondriale en -mikrosomale preparate en toon aan dat komponente in *S. frutescens* ekstrakte verskil spektra induseer en tipe I verskil spektra van natuurlike steroïede inhibeer.

II. Dui die inhiberende effek van *S. frutescens* ekstrakte op die katalitiese aktiwiteit van sitochroom P450-afhanklike-17α-hidroksilase en sitochroom P450-afhanklike-steroïed-21-hidroksilase ensieme in skaapbyniermikrosome aan.

III. Beskryf 'n tegniek om die inhiberende effek van *S. frutescens* op individuele sitochroom P450-afhanklike ensieme – bobbejaan, skaap en mens sitochroom P450-afhanklike-17α-hidroksilase, en bees sitochroom P450-afhanklike-steroïed-21-hidroksilase – in COS1 selle te bepaal.

IV. Demonstreer dat inhibisie van verhoogde glukokortikoïed plasma konsentrasies waargeneem in rotte blootgestel aan kroniese immobiliserende stress, moontlik toegeskryf kan word aan die effek van *S. frutescens* op die sitochroom P450-afhanklike ensieme.
Acknowledgements

I hereby wish to express my sincerest gratitude and appreciation to:

Dr. AC Swart, my promoter, for your tremendous guidance, encouragement, enthusiasm and patience with the project and preparation of this manuscript.

Prof. P. Swart, for his admirable expertise.

David Richfield, for his enthusiastic technical assistance whenever it was required.

Ralie, for her technical assistance, ensuring the smooth progress of my research.

Ilse, for her friendship, motivation and support throughout my university career.

Grant, Karl, Selvan, Wesley, Norbet and Nick, for technical assistance and motivation but most specifically for their contribution to a refreshing, stimulating and enjoyable work environment.

Welma, for all the favours.

The NRF for providing funding.

Sebastian, for your motivation, support, patience, tolerance and affection.

My Parents, without your support, neither Stellenbosch nor this manuscript would have been a reality. Thank you!
Table of Contents

Chapter One
Introduction ........................................................................................................... 1

Chapter Two - Literature Review
The role of adrenal steroidogenesis on the regulation of the HPA-axis and stress
response .................................................................................................................. 5
  2.1 The HPA-axis ...................................................................................................... 5
    2.1.1 The Adrenal Gland ...................................................................................... 8
    2.1.2 Physiology of Cortisol ............................................................................... 10
    2.1.3 Regulation of the HPA-axis ...................................................................... 13
    2.1.4 Adverse effects associated with chronic exposure to elevated cortisol levels. 15
  2.2 Adrenal Steroidogenesis .................................................................................... 19
    2.2.1 Cytochrome P450 ...................................................................................... 21
        Mechanisms of Cytochrome P450 Catalyzed Reactions ............................... 21
        Catalytic Properties of Cytochrome P450 enzymes ............................... 25
    2.2.2 Mitochondrial cytochrome P450 enzymes ................................................. 30
    2.2.3 Microsomal cytochrome P450 enzymes .................................................... 33
    2.2.4 Inhibition of cytochrome P450 enzymes ................................................. 35
  2.3 Summary ............................................................................................................ 37

Chapter Three - Literature Review
Sutherlandia frutescens ......................................................................................... 39
  3.1 Botanical nomenclature, description and distribution .................................... 39
  3.2. Phytomedicines and traditional remedies ....................................................... 41
    3.2.1 Preparation and dosage ............................................................................ 43
    3.2.2 Medicinal potential of traditional remedies - efficacy and safety issues ...... 45
  3.3. Bioactive compounds ...................................................................................... 49
3.4. Pharmacological effects of *S. frutescens* ........................................... 52
3.5 Summary ........................................................................................................ 56

Chapter Four

Inhibition of steroidogenic P450 enzymes by *S. frutescens* ....................... 58

4.1 Introduction ..................................................................................................... 58

4.2 Materials and Methods .................................................................................. 59
  4.2.1 Materials .................................................................................................... 59
  4.2.2 Preparation of *S. frutescens* extracts ..................................................... 60
  4.2.3 Preparation of adrenal mitochondria and microsomes ........................... 61
  4.2.4 Determination of cytochrome P450 concentration ................................ 62
  4.2.5 Spectral binding assays .......................................................................... 63
    Assay A: Inhibitor-induced difference spectra ............................................ 63
    Assay B: Inhibition of steroid-induced difference spectra .......................... 63
  4.2.6 Steroid conversion assays ...................................................................... 64
    Assay A: steroid conversion assays in adrenal microsomes ....................... 64
    Assay B: steroid conversion assays in COS1 cells ..................................... 65
  4.2.7 HPLC analysis of steroid metabolites .................................................... 68
  4.2.8 Liquid chromatography-Mass spectrometry of *S. frutescens* extracts ...... 69
  4.2.9 Statistical analysis .................................................................................. 69

4.3 Results ............................................................................................................. 70
  4.3.1 Inhibition of cytochrome P450 enzymes by *S. frutescens* extracts ........ 72
    4.3.1.1 Spectral assays ................................................................................ 72
    4.3.1.2 Inhibition of steroid conversion ...................................................... 74
    4.3.1.3 Liquid chromatography-Mass spectrometry of *S. frutescens* extracts . 82
  4.3.2 Inhibition of cytochrome P450 enzymes by bioactive compounds in *S.
    frutescens ................................................................................................. 84
    4.3.2.1 Spectral assays ................................................................................ 84
    4.3.2.2. Inhibition of steroid conversion .................................................... 87
  4.3.3 Inhibition of cytochrome P450 enzymes by *Sutherlandia* tablets ........ 87
4.4 Discussion ........................................................................................................................................ 88

Chapter Five
The Effect of *Sutherlandia frutescens* on steroidogenesis: Confirming indigenous wisdom ................................................................. 94

Chapter Six
Conclusion ........................................................................................................................................ 101

References ........................................................................................................................................ 105
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>11βHSD</td>
<td>11-β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>17-OH-PREG</td>
<td>17α-hydroxypregnenolone</td>
</tr>
<tr>
<td>17-OH-PROG</td>
<td>17α-hydroxyprogesterone</td>
</tr>
<tr>
<td>3βHSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>A4</td>
<td>androstenedione</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Ad⁺</td>
<td>reduced adrenodoxin</td>
</tr>
<tr>
<td>Ad⁻</td>
<td>oxidized adrenodoxin</td>
</tr>
<tr>
<td>ADX</td>
<td>adrenodoxin</td>
</tr>
<tr>
<td>ADXR</td>
<td>adrenodoxin reductase</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AP</td>
<td>activator protein</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3'5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CBG</td>
<td>corticosteroid binding globulin</td>
</tr>
<tr>
<td>COSI cells</td>
<td>transformed African green monkey kidney tumor cells</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CYP101</td>
<td>cytochrome P450 cam</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>cytochrome P450 side chain cleavage</td>
</tr>
<tr>
<td>CYP11B1</td>
<td>cytochrome 11β-hydroxylase</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>aldosterone synthase</td>
</tr>
<tr>
<td>CYP17</td>
<td>cytochrome 17α-hydroxylase/17,20 lyase</td>
</tr>
<tr>
<td>CYP21</td>
<td>cytochrome 21-hydroxylase</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detection</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DOC</td>
<td>deoxycorticosterone</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>ESMS</td>
<td>electrospray mass spectrometry</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FMLP</td>
<td>L-formyl-L-methionyl-L-leucyl-L-phenylalanine</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoproteins</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl piperazine ethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPA-axis</td>
<td>hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography / mass spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoproteins</td>
</tr>
<tr>
<td>MAPK</td>
<td>microtubule-associated protein kinase</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>nGRE</td>
<td>negative glucocorticoid response element</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PREG</td>
<td>pregnenolone</td>
</tr>
<tr>
<td>PROG</td>
<td>progesterone</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SARs</td>
<td>structure-activity relationship studies</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone binding globulin</td>
</tr>
<tr>
<td>STAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>Th1 and Th2</td>
<td>helper T lymphocyte</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>tetrathorbol acetate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>
Chapter One

Introduction

The popularity of herbal remedies and medicinal plants has escalated seeing a 380 % rise in the use of herbal products in the United States from 1990 till 1997 [Eisenberg et al., 1998]. In South Africa, *Sutherlandia frutescens* is a highly regarded medicinal plant that has been used for decades to treat stress and illnesses related to stress [Van Wyk and Wink, 2004; Van Wyk et al., 1997]. Although most of the therapeutic claims are anecdotal reports, recently there have been many scientific investigations into the physiological effects of *S. frutescens* to support these claims. The scientific validation of the biological effects of medicinal plants is imperative to ensure the safety and efficacy of its continued use.

Conventional medicines and xenobiotics are metabolized in the liver by the cytochrome P450 system. St John’s wort and Ginkgo Milk Thistle, two commonly used herbal remedies, have been found to interact with cytochrome P450 3A4, a liver P450 enzyme [De Smet, 2002; Marcus and Grollman, 2002]. Such reports suggest that the use of herbal remedies together with conventional medicine may compromise, delay or replace an effective form of conventional medicine, highlighting the importance of the regulation of the use of herbal remedies. Since herbal remedies are able to interact with the liver P450 enzymes, it may be possible that they also interact with the steroidogenic cytochrome P450 enzymes of the adrenal. The steroidogenic P450 enzymes catalyse, amongst others, cortisol biosynthesis, the major stress hormone [Lewis, 1996, 2001; Ruckpaul and Rein, 1990]. Thus the aim of this study was to investigate the potential stress relieving properties of *S. frutescens* by determining the possible interaction with steroidogenic cytochrome P450 enzymes, resulting in an inhibition of cortisol biosynthesis.

Stress, be it psychological or physical, activates the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA-axis leads to an elevation in the concentration of circulating glucocorticoids, cortisol and corticosterone, and is discussed in Chapter 2. Glucocorticoids are responsible for maintaining the homeostasis of the central nervous system, metabolism and immune function. The glucocorticoids released in response to stress also form part of the
negative feedback system regulating the HPA-axis. In chronic stress, the negative feedback regulatory mechanism is overridden and cortisol levels are therefore permanently elevated [Huizenga et al., 1998]. Long-term exposure to elevated glucocorticoids has been associated with numerous clinical conditions — hypertension, cardiovascular disease, diabetes, melancholic depression, osteoporosis, cholesterolemia, visceral fat syndrome and suppression of the reproductive system, many of which result from a hormone imbalance [Chrousos and Gold, 1998]. A mechanism to alleviate these negative effects arising from the chronic stimulation of the HPA-axis could employ therapies aimed at decreasing plasma glucocorticoid levels.

Since the cytochrome P450 enzymes are responsible for the biosynthesis of cortisol in the adrenal gland, inhibition of these enzymes might be a valuable therapy to treat maladies associated with elevated plasma glucocorticoid levels. Illnesses associated with the dysfunctioning of the adrenal are treated with cytochrome P450 inhibitors to lower glucocorticoid and other plasma steroid concentrations [Child et al., 1976; Contreras et al., 1985; McCance et al., 1987; Nieman, 2002; Verhelst et al., 1991]. The cytochrome P450 enzymes catalyse adrenal steroid biosynthesis and are considered complex monoxygenase enzyme systems and are discussed in detail in Chapter 2 [Lewis, 1996]. These enzymes are membrane bound heme proteins which when reduced by carbon monoxide and dithionate, demonstrate a characteristic Soret peak at 450 nm. The enzymes incorporate one atom of molecular oxygen into their substrate and the other into water. The reaction requires two electrons which are acquired from NADPH and migrate via redox partners to the cytochrome P450 heme iron. The cytochrome P450 enzymes employ two different electron transfer chains, dependent on the location of the enzyme in the cell [Lewis, 1996, 2001; Ruckpaul and Rein, 1990]. Cytochrome P450 enzymes present in the endoplasmic reticulum acquire their electrons from cytochrome P450 reductase while in the mitochondria, the electron transfer chain comprises adrenodoxin and adrenodoxin reductase [Takemori and Kominami, 1984]. Adrenodoxin shuttles the electrons to the P450 enzyme one at a time from adrenodoxin reductase. The complexity of the reactions catalysed by the P450 enzymes highlights the many possible inhibitory mechanisms that may be employed. For example, inhibitors may bind to the enzyme and therefore interfere with natural substrate binding, or the binding of the redox partners which would obstruct the transport of electrons. Although the steroidogenic P450 enzymes are highly specific for their natural steroid
substrates, inhibitors binding to the expressed cytochrome P450 enzymes usually inhibit all the P450 enzymes in the steroidogenic pathway.

*S. frutescens*, one of the 3000 plants used for medicinal purposes in South Africa, is discussed in Chapter 3. Decoctions of the stems and leaves of *S. frutescens* have traditionally been used to treat a multitude of physiological afflictions including the symptoms of stress and is currently considered the most profound adaptogenic tonic [Van Wyk and Wink, 2004; Van Wyk *et al.*, 1997]. Anecdotal evidence of *S. frutescens* improving the quality of life has resulted in the marketing of this medicinal plant in tablet form even though there is very little scientific evidence supporting therapeutic claims or possible implications of its long-term use. Safety and efficacy issues surrounding herbal remedies and medicinal plants are addressed. The medicinal value of plants is usually attributed to secondary metabolites and their concentrations which are, in turn, determined by the environment. Traditional healers have identified, by trial and error, not only which plants have medicinal properties but also which parts of the plants contain the active compounds required for healing purposes. Some of the compounds that have been identified in *S. frutescens* include L-canavanine, pinitol, gamma-aminobutyric acid, various flavonoids and triterpenoids which may contribute toward some of the positive anecdotal effects that have been reported. Current research into the pharmacological effects of *S. frutescens* and claims of anti-HIV and anti-cancer properties, which sparked scientific investigations into the medicinal potential of *S. frutescens*, are presented.

In Chapter 4 the investigation into the bioactivity of *S. frutescens* is presented. Aqueous and methanol extracts of *S. frutescens*, bioactive compounds as well as commercially available tablets were investigated for possible interactions with the steroidogenic P450 enzymes. Due to the unique spectral properties of the cytochrome P450 enzymes, interactions with these enzymes can be monitored by UV spectrometry. Two spectral assays were carried out to determine if the test components could firstly, bind to the P450 enzymes and secondly, influence the binding of natural steroid substrates. The influence of the *S. frutescens* on the catalytic activity of the P450 enzymes was subsequently investigated *in vitro* and in COS 1 cells as was the inhibitory effect of test components on the catalytic activity of mitochondrial and microsomal cytochrome P450 enzymes. These assays were augmented by investigations of *S. frutescens*’ inhibitory effects on 17α-hydroxylase/17,20 lyase and 21-hydroxylase expressed in COS1 cells.
The anti-stress properties of *S. frutescens* may be attributed to more than just one compound present in the plant which could influence the release and/or synthesis of the glucocorticoids at various levels of the HPA-axis. In order to scientifically appraise the stress relieving properties of *S. frutescens*, plasma glucocorticoid concentrations were monitored in rats subjected to chronic immobilization stress, in the absence and presence of administered *S. frutescens* [Smith and Myburgh, 2004]. Chapter 5 reports that rats treated with *S. frutescens* exhibited lower plasma glucocorticoid concentrations in response to chronic stress compared to the control rats receiving a placebo. The plant may thus alleviate the symptoms of stress by decreasing the circulating glucocorticoid levels. The mechanism of action may be, amongst others, through the inhibition of the steroidogenic cytochrome P450 enzymes which catalyse the biosynthesis of glucocorticoids.

Chapter 6 summarises the findings and concludes this study of the investigations into the bioactivity of *S. frutescens*. 
Chapter Two

The role of adrenal steroidogenesis in the regulation of the HPA-axis and stress response

Stress can be defined as any perturbation of homeostasis and is stimulated by a stressor. The stressor, which is defined as an event that elicits stress, may be either psychological stressors like major life events, trauma and environmentally related factors, or physical stressors which include pathogens and toxins. Both types of stressors activate the hypothalamic pituitary adrenal (HPA) axis stimulating the increased production of cortisol by the adrenal gland. Acute stress results in temporarily elevated cortisol plasma levels while chronic stress is associated with permanently elevated cortisol concentrations.

It has been known for thousands of years that psychological stress can affect the outcome of disease and was first recorded by Galen in 200AD [Dunn, 1996]. The detrimental effects of stress on the outcome of disease may be attributed to chronic elevated cortisol levels. It has been proposed that chronic exposure of physiological systems to repeated elevation of cortisol could impact markedly on homeostasis and health [Chrousos and Gold, 1998; Reiche et al., 2004; Seeman et al., 1997]. The stress response, the biosynthesis of cortisol in the adrenal gland catalysed by the cytochrome P450 enzymes as well as the effects of stress, will be explored in this chapter.

2.1 The HPA-axis

The HPA-axis is activated in response to either physical or psychological stress, invariably leading to elevated levels of the glucocorticoid hormone, cortisol, secreted from the adrenal cortex [Munck et al., 1984; Traustadóttir et al., 2005; Vander et al., 2001]. The function of the HPA-axis is to maintain basal and stress related homeostasis of the central nervous system, cardiovascular, metabolic and immune function [Chrousos, 1998]. Physical stress includes fever, surgery, burn injury, hypo- and hyperglycaemia, hypo- and hypertension and exercise.
Psychological stress is different to physical stress in that there is no clear beginning or end and it is not linked to an increase in metabolic demand. It is not only the HPA axis but also the sympathetic nervous system (noradrenergic system controlling autonomic outputs) which is activated by stress as illustrated in Figure 1 [Chrousos and Gold, 1998; Haas, 2001; Moynihan, 2003; Reiche et al., 2004; Sved et al., 2002; Vander et al., 2001; Young et al., 2005].

Neurosensory signals are ultimately processed in the nucleus of both the hypothalamus and the locus coeruleus-noradrenergic centre. The paraventricular nucleus (PVN) of the hypothalamus is first to respond to the positive signal for the secretion of cortisol. Specialized neurons of the PVN project into the median eminence of the hypothalamus. Axonal processes subsequently form special junctions with the capillaries of the pituitary. The PVN produces, corticotrophin releasing hormone (CRH), a neuropeptide, which is carried by the portal vein to the anterior pituitary stimulating corticotroph cells to produce the complex pro-opiomelanocortin (POMC) proteins [Vale et al., 1981]. POMC is cleaved to form adrenocorticotropic hormone (ACTH), beta-endorphin and other peptide hormones [Guillemin et al., 1977]. When the ACTH reaches the adrenal cortex, the rate of cortisol biosynthesis and secretion is increased, and cortisol is subsequently released into the blood stream. During times of stress there is not only an increase in CRH, ACTH and cortisol, but also a significant increase in cholesterol levels since it is the substrate for cortisol biosynthesis in the adrenal glands.
Figure 1. Stress response and the interactions between the nervous, endocrine and immune system. The hypothalamic factors regulate the pituitary cells and the sympathetic nervous system. The glucocorticoids specifically cortisol, inhibit the production of corticotrophin-releasing factor (CRF, corticotrophin releasing hormone CRH) and adrenocorticotropic hormone (ACTH). + stimulation, - inhibition [reproduced from Reiche et al., 2004].

Activation of the sympathetic nervous system by either physical or psychological stress, results in the secretion of the catecholamines, epinephrine and norepinephrine, from the adrenal medulla into the blood (Figure 1). This response is more commonly known as the fight-or-flight response where an immediate physical response is required to prevent bodily harm. Epinephrine increases the rate and depth of respiration, mobilizes glucose as a source of energy and increases the heart rate and cardiac output. Norepinephrine is a potent vasoconstrictor, maintains blood pressure and also increases the rate and depth of respiration [Reiche et al., 2004; Saunders, 2003; Vander et al., 2001]. Receptors for the catecholamines are present in smooth muscle, cardiac muscle,
adrenal gland cells and specific central nervous system neurons [Vander et al., 2001]. The locus coeruleus, a nucleus in the brain stem pons, contains noradrenergic neuronal cell bodies. These neurons provide a stimulatory noradrenergic input to the PVN activating the HPA axis [Young et al., 2005; Sved et al., 2002]. Smagin et al. (1995) have suggested that norepinephrine may regulate the release of CRH from the hypothalamus. Furthermore, the catecholamines also regulate the secretion of hormones from the adrenal as well as the expression of some cytochrome P450 enzymes responsible for catalyzing the biosynthesis of adrenal steroids [Smagin et al., 1995].

The hippocampus and amygdale can influence the HPA axis via the PVN [Felman et al., 1995]. The hippocampus is a malleable brain structure that is sensitive to the effects of stress and trauma and is important for certain types of memory and learning. During hippocampal formation, high levels of adrenal steroid receptors are expressed [McEwan, 2005]. It is known that adrenocortical hormones have a wide variety of effects in the brain and these hormones have been linked to dendritic shortening [De Kloet et al., 1998; McEwan, 2003; McEwan, 2004].

2.1.1 The Adrenal Gland

Activation of the HPA-axis results in the release of cortisol from the adrenal glands. The adrenal glands lie above the kidney and express the enzymes responsible for the production of steroids - 3β-hydroxysteroid dehydrogenase (3βHSD) enzyme and five cytochrome P450 enzymes; cytochrome P450 side chain cleavage (CYP11A1), 11β-hydroxylase (CYP11B1), aldosterone synthase (CYP11B2), 17α-hydroxylase/17,20 lyase (CYP17) and 21-hydroxylase (CYP21). The adrenal gland can be divided into two distinct zones; the cortex and medulla, where the medulla occupies the centre of the gland and makes up approximately one quarter of the adrenal mass. The medulla contains two distinct cell types, the norepinephrine secreting cells, which are small, dense granules and the epinephrine secreting cells, which are larger, less dense granules. The medulla chromaffin cells have been identified in the cortical region and similarly cortical cells have been identified in the medulla. This distribution of the cells within the adrenal provides extensive contact areas, enhancing paracrine signalling which influences adrenal steroidogenesis. The catecholamines secreted from the medulla stimulate the secretion of cortisol, aldosterone
and androstenedione from the adrenocortical cells. Furthermore, the catecholamines have stimulatory effects on the transcription of some cytochrome P450 genes [Guse-Behling et al., 1992].

The adrenal cortex consists of three zones; zona glomerulosa, zona fasciculata and the zona reticularis (Figure 2). The mineralocorticoids are produced and secreted by the glomerulosa cells. Androgens and glucocorticoids are produced in the remaining two zones. CYP17 is not expressed in the zona glomerulosa, but is expressed in the zona fasciculata and to a lesser extent in the zona reticularis. CYP21 is located in all three zones of the adrenal cortex [Inano et al., 1969]. Cortisol production takes place primarily in the zona fasciculata but also in the zona reticularis while it is not produced in the zona glomerulosa due to the lack of CYP17 expression in this zone. Aldosterone synthase (CYP11B2) is expressed only in the zona glomerulosa and aldosterone is therefore the only hormone of physiological significance secreted by the zona glomerulosa.

Both the medulla and adrenal cortex are innervated by a rich nerve supply that synapses directly with the steroid producing cells [Vinson et al., 1994]. Both endocrine and neural activities control adrenal activity. Many different neurotransmitters have been shown to be involved in relaying signals to the endocrine steroid producing cells. The catecholamines and acetylcholine...
are two of the neurotransmitters secreted to the adrenal cortical cells. The regulation of this neural component that controls adrenal steroidogenesis is complex and not well understood [Vinson et al., 1994] but its role is most likely involved in the fine-tuning of adrenal steroidogenesis.

2.1.2 Physiology of Cortisol

The glucocorticoids, specifically cortisol in humans and corticosterone in rats, which are released in response to stress, play an important role in the stress response. Cortisol influences organic metabolism in mobilising fuels by increasing plasma concentrations of amino acids (source of glucose via gluconeogenesis and available for tissue repair if damage was incurred), glucose, glycerol and free fatty acids. Cortisol has also been shown to increase vascular reactivity [Munck et al., 1984; Seeman et al., 1997; Traustadóttir et al., 2005] and has immunosuppressive effects on lymphocytes and macrophages. Glucocorticoids decrease the production of many cytokines, mediators of inflammation, and decrease effects of some inflammatory molecules on the target tissue [Chrousos and Gold, 1998; Hässig et al., 1996; Kunz-Ebrecht et al., 2003; Maes et al., 1998; Moyniham, 2003; Reiche et al., 2004; Vander et al., 2001]. These effects of cortisol protect against damage from excessive inflammation [Vander et al., 2001]. The primary function of the glucocorticoids in the stress response is therefore to protect the body from its own defence mechanisms. The cortisol response is vital since without it an individual may experience circulatory failure and possible death [Munck et al., 1984; Vander et al., 2001].

Basal cortisol concentrations vary in a circadian rhythm as illustrated in Figure 3. Cortisol concentrations peak after waking, with a 50-70 % increase in cortisol levels within half an hour of waking, and are lowest just before sleep [Pruessner et al., 1997]. The basal levels of cortisol range between 165-690 nmol/l. Unbound cortisol is biologically active and 3.8 % of plasma cortisol is normally unbound [Pruessner et al., 1997]. Under normal conditions, the average plasma cortisol concentration in human adults at 8 a.m. is 358 nmol/l at a secretion rate of 15 mg/day while during severe stress the maximal rate of cortisol secretion is 300-400 mg/day [Saunders, 2003]. Chronic stress results in sustained increased cortisol plasma concentrations (Figure 3) [Chrousos, 1998; Chrousos and Gold, 1998; Weber et al., 2000]. Increased
susceptibility to infectious disease has been associated with persistent elevations of cortisol concentrations. Cortisol concentrations are related to gender as the cortisol responsivity in females changes during the menstrual cycle. On the other hand, cortisol concentrations are not related to age, weight, smoking status, or alcohol consumption (the night prior sampling) [Pruessner et al., 1997]. Interestingly another study [Traustadóttir et al., 2005] has indicated that amongst unfit women, ageing is associated with increased reactivity of the HPA-axis, indicating that exercise might be an effective way of modifying neuroendocrine changes associated with aging.

Figure 3. Circadian pattern of cortisol secretion in nonstressed (NS solid line) and chronically stressed (CS dotted line) individuals [reproduced from Chrousos and Gold, 1998]

In humans, steroid hormones circulate in the plasma mainly bound to proteins — sex hormone binding globulin (SHBG), corticosteroid binding globulin (CBG) and albumin [Dunn et al., 1981]. SHBG and CBG are both highly specific and have low binding capacities whereas; albumin is a non-specific steroid binding protein with a high binding capacity, functioning as a reservoir for circulating steroids. Deoxycortisol, corticosterone and cortisol are the main physiological ligands for CBG and all bind with similar binding affinity while the androgens bind with low affinity [Dunn et al., 1981]. SHBG has a high affinity for the androgens dehydroepiandrosterone (DHEA). Of the average 400 nM cortisol present in the plasma, approximately 89.7 % is bound to the CBG and 6.3 % is bound to albumin while 3.8 % is unbound [Dunn et al., 1981; Louw, 1998]. The binding of cortisol to the plasma proteins
increases its half-life. The plasma concentrations of the binding proteins fluctuate independently throughout life for development and maturation of cellular functions, but may also be altered in specific disease states. The free versus bound ratio is not constant and free cortisol can vary up to 200 % from the minimum value when the total CBGs only decrease a maximum of 25 % [Dunn et al., 1981; Saunders, 2003]. When the CBG concentration is increased, there is an increase in cortisol bound to the CBG and a decrease in the percent of unbound cortisol to alleviate the suppressing effects of glucocorticoids on the release of ACTH from the pituitary [Dunn et al., 1981; Louw, 1998].

The circulating cortisol makes its way through the kidney into urine as well as through the parotid gland into saliva. Furthermore, cortisol crosses the blood brain barrier where it is able to reach all parts of the central nervous system (CNS). Cortisol enters the cerebrospinal fluid via the choroids plexus to gain access to the regulatory sites on the amygdala, hippocampus and hypothalamus. Access to the anterior pituitary is gained through systemic circulation [Saunders, 2003]. Munck et al. (1984) stated that the physiologically important effects associated with glucocorticoids, are not simply a consequence of primary action exerted by the steroid on the target tissue, but rather the secondary effect, which employs various intercellular mediators resulting in the desired response. The mechanisms through which glucocorticoids act involve the glucocorticoid receptors (GRs) and the mineralocorticoids receptors (MRs) and may require RNA and protein synthesis [Munck and Brinck-Johnson, 1967]. Since the adrenal steroids have receptors in all nucleated cell types, cortisol is able to cross membranes and to bind to receptors in the cytoplasm and enter the nucleus, where its main function is to either inhibit or induce the transcription of a wide range of genes [Saunders, 2003]. Access to the receptors is regulated by the circulating concentration of steroid hormones, corticosteroid binding globulins, the density of the two receptor types and by the enzyme 11-β-hydroxysteroid dehydrogenase (11βHSD) [Weber et al., 2000]. Cortisol is converted to its inactive form cortisone and visa versa by 11βHSD in the liver. The liver is the principal site for glucocorticoid catabolism where cortisol is broken down and conjugated to glucuronic acid, a soluble product which is excreted in the urine. The liver is responsible for degrading 15 % of the secreted cortisol, which is excreted in the stool. The CYP3A family of enzymes is the most abundant group of P450 enzymes in the liver. The CYP3A enzymes metabolize fat soluble hormones including cortisol and estrogens and these enzymes are thus responsible for clearing cortisol from the system. Cortisol is converted to 6β-
hydroxycortisol by CYP3A and the measurement of urinary 6β-hydroxycortisol has widely been used as a non-invasive clinical test to detect cytochrome P450 induction. Abel et al. (1992), however, demonstrated the highly complex and variable hepatic metabolism of cortisol by identifying a myriad of metabolites of cortisol including 6α- and 6β-hydroxycortisol, 20β-dihydroxycortisol, 20β-dihydroxy cortisol, cortisone and 3α- and 3β-tetrahydrocortisone and others, suggesting that the analysis of CYP3A activity in man is limited when only 6β-hydroxycortisol excretion is measured.

DHEA is another major steroid produced by the adrenal and acts as a functional antagonist of cortisol. The concentration of DHEA in human adult plasma is 0.01 μM to 0.02 μM [Kalimi et al., 1994]. DHEA is a C19 steroid classified as an androgen, which has anti-glucocorticoid effects, namely, anti-stress, anti-diabetic, anti-ageing. DHEA has been shown to boost the immune system and improves memory by protecting brain neurons, thereby blocking the deleterious effects of cortisol during stress [Kalimi et al., 1994]. DHEA levels do not increase in response to acute stress. However, Heuser et al. (1998) found that in severely depressed patients, which is understood as a chronic stress condition, both cortisol and DHEA were hyper secreted in a circadian pattern, suggesting a possible adaptive cortisol-antagonizing effect in chronic stress [Heuser et al., 1998].

2.1.3 Regulation of the HPA-axis

Three primary structures regulate the secretion of adrenocortical glucocorticoids by negative feedback, namely, the pituitary gland, the hypothalamus and the hippocampus [Jacobson, 1991; Young et al., 2005]. The hypothalamus receives a wide array of both chemical and electrical stimuli to sense the homeostasis of the organism. In response to stimuli, the hypothalamus generates both chemical and electrical signals and is thus the centre for integrating the nervous and endocrine systems. The activity of the adrenocortical system increases in a circadian rhythm, and more significantly so in response to stress [Jacobson and Sapolsky, 1991; Lovallo, 2000].

Sites for the negative feedback are mostly identified by the presence of corticosteroid receptors. GRs are abundant in the limbic system (lateral septum), central amygdale, dentate gyrus of the
hippocampus and the noradrenergic neurons in the locus coeruleus. These glucocorticoid receptors are targets for cortisol and possible targets for negative feedback on stress-activated brain mechanisms [Reul and De Kloet, 1985]. Both glucocorticoid- and mineralocorticoid receptors have been located in the hippocampus which expresses the highest levels of these receptors compared to other regions in the brain. The hippocampus is therefore considered the focal point for the regulation of the activity of the HPA-axis. GRs can bind only glucocorticoids, whereas MRs, which have a higher affinity for glucocorticoids, can bind both glucocorticoids and mineralocorticoids [Reul and De Kloet, 1985]. The role of the hippocampus in regulation of the HPA-axis was reviewed by Jacobson and Sapolsky (1991) and they suggest that the hippocampus regulates the activity of the HPA, most significantly by inhibiting the secretion of ACTH [Dallman et al., 1987; McEwan, 1997; Saunders, 2003].

ACTH secreted by corticotropes in the anterior pituitary drives the release of cortisol from the adrenal cortex. ACTH secretion is regulated by factors (CRH, vasopressin, oxytocin, epinephrine) secreted from neurons in the hypothalamo-hypophysial portal circulation [Jacobson and De Kloet, 1991; Plotsky, 1985]. The activity of the HPA-axis is reduced by suppression of the CRH gene by mechanisms sensitive to cortisol concentration [Jacobson and De Kloet, 1991; Munck et al., 1984]. Cortisol therefore has a negative feedback effect on the release of CRH from the hypothalamus and is illustrated in Figure 1. In addition, POMC (which is cleaved to form ACTH and β-endorphin) biosynthesis is rapidly reduced as a result of decreased CRH, resulting in decreased ACTH levels. The POMC gene is an additional negative feedback target as it contains a negative glucocorticoid response element (nGRE) to which cortisol (bound to the GR) binds, inhibiting POMC synthesis which, in turn, inhibits the production of ACTH [Drouin et al., 1993; Slabbert, 2003]. Regulation of adrenocortical activity also takes place within the adrenal (intra-adrenal activity) as ACTH is not only derived from the anterior pituitary but is also produced by the adrenal [Markowska et al., 1993]. Furthermore, CRH expression has been shown in the adrenal gland and has direct effects on the activity of the adrenal [Bornstein et al., 1990]. ACTH has an acute and chronic effect on the adrenal resulting in the stimulation of steroidogenesis and growth. An acute response stimulates steroidogenesis through a steroidogenic acute regulatory (StAR) protein mediated increase in cholesterol. StAR stimulates the mobilization of cholesterol from lipid stores to the inner mitochondrial membrane and thus increases the availability of cholesterol for steroid hormone biosynthesis. The chronic effect of
ACTH increases the synthesis of all steroidogenic cytochrome P450 enzymes and adrenodoxin reductase. ACTH binds to a G protein-coupled melanocortin-2 receptor on adrenocortical cells which activates adenylate cyclase producing cAMP from ATP. cAMP-dependent protein kinase (PKA) is subsequently activated resulting in the release of catalytic subunits which activate the synthesis and activity of enzymes by phosphorylation [Lewis, 2001; Lisurek and Bernhardt, 2004; Waterman and Bischof, 1997]. Angiotensin II, cytokines and Ca ++, are some of the many additional factors also involved in the regulation.

2.1.4 Adverse effects associated with chronic exposure to elevated cortisol levels

Activation of the stress response by a psychological event is not simply a response to the stimuli. The reaction to the stressor is determined by the individual’s perception of, and interaction with the stressor, since individuals respond to similar situations or stress differently [Lovallo, 1997; Saunders, 2003]. The repeated delivery of stress, which includes psychological, chemical or physical stress, may lead to drastic changes in the physiology of neural endocrine, immune and digestive systems [Ha et al., 2003].

Acute stress refers to temporary elevated plasma cortisol levels, after which the cortisol levels and activity of the HPA-axis return to basal conditions. Excessive or chronic stress would result in permanently elevated plasma cortisol levels and the homeostasis would have to be re-established, a phenomenon known as allostasis [Seeman et al., 1997]. Allostasis of the nervous system, HPA-axis, metabolism, cardiovascular and immune system is brought about by the catecholamines and the glucocorticoids. The glucocorticoid hormones, catecholamines and the pro-inflammatory cytokines are the principal messengers for communication between the central nervous system and the immune system [Maes et al., 1998; Reiche et al., 2004]. Young et al. (2005) concluded that the noradrenergic system can influence the magnitude of the HPA-axis response to stress, but in major depression, activation of the HPA-axis appears autonomous of the noradrenergic influence. A prolonged stress response and the sustained activity of the HPA-axis together with persistent increases in cortisol production, have been associated with depression, hypertension, osteoporosis, immunosuppression [Chrousos and Gold, 1998; Hässig et al., 1996; Kunz-Ebrecht et al., 2003; Maes et al., 1998; Moynihan, 2003; Seeman et al., 1997;...
Reiche et al., 2004], visceral obesity and insulin resistance [Hautanen and Adlercreutz, 1993; Rosmond et al., 1998]. There has also been substantial evidence suggesting that prolonged stress results in an increased risk of chronic conditions such as, cardiovascular disease, diabetes and atherosclerosis [Bjorntorp, 1997; Chrousos and Gold, 1998].

Human [Kunz-Ebrecht et al., 2003; Maes et al., 1998] and animal [Sklar and Anisman, 1979] studies have suggested that stress renders these subjects more susceptible to disease and impairs the functioning of the immune system and this topic has been reviewed by many researchers [Chrousos and Gold, 1998; Hässig et al., 1996; Reiche et al., 2004]. Chronic stress significantly suppresses the immune response of blood and spleen lymphocytes, T-cell mitogenesis, phagocytosis, Natural killer (NK) cell activity and production of inflammatory cytokines, interleukin 2 and interferon γ [Chrousos and Gold, 1998; Hässig et al., 1996; Kunz-Ebrecht et al., 2003; Maes et al., 1998; Moynihan, 1989 & 2003; Munck et al., 1984; Reiche et al., 2004]. Studies investigating different stressors and species have shown variable results since the interpretation of the response to stressors vary between humans and/or animals - different aspects of the immune response are inhibited, demonstrating the complexity in communication between the brain, the immune system and behaviour. Different experimental designs of studies and immunological assays, forms of depression assessment and age of individuals are important factors involved in the variability, which have to be taken into account when interpreting the results.

Maes et al. (1998) investigated the effect of psychological stress on the Th-1-like response and found that elevated plasma cortisol levels are associated with an increased response. The Th-1 response involves the secretion of IFN-γ and IL-2, which are involved in cellular immune functions, whereas the Th-2-like response involves the secretion of IL-4 and IL-5 thus enhancing humoral immune reactions associated with B cells. IL-4 and IL-10 suppress Th1 effector functions [Maes et al., 1998; Hässig et al., 1996]. Increased plasma cortisol levels result in a long-lasting suppression of cellular immune reactions associated with T-cells and thus increase in susceptibility to infection [Hässig et al., 1996]. Reiche et al. (2004) summarized the effects of the stress hormones on the immune system illustrated in Figure 4.
The progression of HIV infection is also accompanied by alterations in the production of the adrenal steroids [Clerici 1997]. HIV patients exhibit increased cortisol and decreased DHEA plasma levels [Chrousos 1998, Clerici et al., 1997]. It has been reported that DHEA can act as a functional antagonist of the immunoregulatory activities of cortisol [Heuser et al., 1998]. Elevated cortisol levels inhibit IL-1 and the transcription of IL-2 and IFNγ genes but not the transcription of IL-4 [Clerici et al., 1997]. Thus, individuals experiencing chronic stress may be more susceptible to disease as a result of the inhibition of the immune response.

Figure 4. Systemic effects of glucocorticoids and catecholamines on the immune system. NK, natural-killer cells; MO, macrophage; Th1, T-helper lymphocyte type 1 cells; Th2, T-helper lymphocyte type 2 cells; TNF, tumor necrosis factor [Reproduced from Reiche et al., 2004].
Cancers associated with DNA tumor virus are more likely to be affected by psychological stress than cancers induced by chemical carcinogens. It is possible that stress and depression foster tumor expression since increased cortisol production associated with stress is able to inhibit various aspects of the immune system. Stress therefore compromises some of the important effectors of the immune response against tumors [Munck et al., 1984, Reiche et al., 2004]. Kiecolt-Glaser et al. (1985) concluded depressed or stressed patients showed significantly decreased DNA repair of damaged DNA as compared to less stressed patients. Levav et al. (2000) showed that the incidence of cancer was increased in parents of accident victims, as compared to parents who had not lost offspring. After a twenty year follow up on the patients it was noted that the risk of death (as opposed to the progression of cancer) was increased if the patient was diagnosed before their loss [Levav et al., 2000]. These findings indicate that stress may render an individual more susceptible to cancer and, in addition, severe stress experienced after being diagnosed with cancer, could result in increased progression of the disease and thus increased mortality.

The stress hormones, which include the glucocorticoids and catecholamines, can also modulate brain function by changing the structure of the neurons in the hippocampal formation [McEwan, 2005]. The dendrite gyrus, within the hippocampus is most vulnerable to some types of acute and chronic stressors. These stressors suppress neurogenesis or cell survival in the dendrite gyrus [McEwan, 1997, 2003 & 2005]. The CA3 region of the hippocampus is connected to the dendrite gyrus and it has been documented that chronic stress causes retraction and simplification of the dendrites in this region. Thus far the remodelling of the hippocampus which occurs in response to stress has been found to be largely reversible over a three week period [McEwan, 2003]. However, it is possible that sustained stress may cause more permanent damage. Furthermore it is important to note that the hippocampus may be more vulnerable in sustained stress situations if the mechanisms activated in response to stress were not in place [McEwan, 2003 & 2005]. The prefrontal cortex and amygdale are also influenced by stress, since repeated stress has resulted in dendritic shortening in the prefrontal cortex and conversely, dendritic growth in neurons of the amygdale [McEwan, 1997, 2003 & 2005]. Roelofs et al. (2005) have recently suggested that increased cortisol levels are also associated with diminished active approach avoidance behaviour with subjects showing slower avoidance actions to threatening stimuli in times of increased stress.
Chronic restraint stress has a potent significant effect on the gene expression profile in rat liver [Ha et al., 2003]. Genes altered most significantly included those involved in lipid metabolism and detoxification. Chronic stress suppressed the uptake of glucose from peripheral tissue and the immune response. In addition, fat stored energy was mobilized and gluconeogenesis stimulated. Although the exact mechanism whereby stress stimuli was able to trigger these metabolic and physiological changes, remains uncertain, these results suggest that these changes may be associated with the altered gene profile [Ha et al., 2003].

In summary, evidence from animal and human studies strongly suggests that persistent stress and depression result in impairment of the immune response as well as an increased susceptibility to the onset of cancer and other diseases. There are many conflicting reports due firstly, to the diversity of the experimental approaches and secondly, the diversity of individuals studied. Although these adverse effects of stress cannot as yet be attributed to a specific factor of the stress response, cortisol does appear to be a key player [Dimsdale and Herd, 1982; Segerstrom, 2003]. Cortisol biosynthesis takes place in the adrenal gland and the reactions are catalysed by the steroidogenic cytochrome P450 enzymes.

2.2 Adrenal Steroidogenesis

The adrenal gland secretes the mineralocorticoid, glucocorticoid and androgenic steroid hormones. Mineralocorticoids regulate the water and sodium/potassium balance in the body. Mineralocorticoid action results in the absorption of sodium from urine, sweat, saliva and gastric juices. Glucocorticoids play a significant role in the regulation of protein, carbohydrate and lipid metabolism, while the androgens are required for the functional development of secondary sex characteristics. The reactions involved in steroid hormone biosynthesis are primarily of the monooxygenase type, catalyzed by various cytochromes P450 enzymes [Ruckpaul and Rein, 1990]. The catalytic reactions involved in corticosteroid biosynthesis include: hydroxylations, C-C bond cleavages, dehydrogenation, isomerization and oxidation reactions. Reactions in the steroid hormone biosynthesis pathway (Figure 5) are catalyzed in the mitochondria and endoplasmic reticulum of the adrenal cortex, by the cytochrome P450 enzymes and 3β-
hydroxysteroid dehydrogenase (3βHSD). The mitochondria contain cytochrome P450 side chain cleavage (CYP11A), cytochrome-11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2), which are associated with the inner mitochondrial membrane. Cytochrome P450 21-hydroxylase (CYP21) and cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17) are embedded in the endoplasmic reticulum [Hall, 1986; Lisurek and Bernhardt, 2004; Machino et al., 1969; Payne and Hales, 2004; Ruckpaul and Rein, 1990; Takemori and Kominami, 1984]. The metabolic intermediates move back and forth between the endoplasmic reticulum and mitochondria with the initial and final reactions of steroidogenesis taking place in the mitochondria. The four major P450 species are integral membrane proteins, embedded in the organelles and their substrate binding site faces the lipid phase.

Figure 5. Steroid biosynthesis in the adrenal cortex [reproduced from Lisurek and Bernhardt, 2004].
2.2.1 Cytochrome P450

Garfunkel (1958) and Klingenburg (1958) were the first to discover the heme-thiolate proteins and in 1964 [Omura and Sato, 1964] the carbon monoxide binding pigment of liver and adrenal microsomes was named cytochrome P450, and shown to be monooxygenases involved in the oxidation of drugs and steroids. These cytochrome P450 enzymes, most abundant in the liver, are predominantly found in the endoplasmic reticulum and mitochondria. Levels of these enzymes in the liver as well as in other organs are variable and can differ as a result of chemical induction, genetic variation and dietary habits, age, gender, etc [Lewis, 1996 & 2001]. The cytochrome P450 enzymes are distributed in all five kingdoms, with the exception of a primitive bacterial species. The enzymes are present in a variety of organs and tissues — kidney, the lungs, the skin, the adrenal gland, the spleen, ovaries, testis, placenta and brain. The cytochrome P450 enzymes differ from the other cytochromes (cytochrome a, b, c) in that their purpose is not solely for electron transfer through a redox potential gradient, but rather for the activation of a dioxygen molecule with the subsequent insertion of a single oxygen atom into the substrate [Lewis, 1996 & 2001; Ruckpaul and Rein, 1990]. Peroxygenation, hydroxylation, epoxidation, deamination, desulfuration, dehalogenation and reduction are some of the various reactions catalyzed by cytochrome P450 enzymes. The reactions have a wide range of physiological functions including drug metabolism, detoxification of carcinogens and xenobiotics, metabolism of fatty acids, bile acids, Vitamin D and the metabolism of steroids. The interaction of cytochrome P450 with xenobiotics is a detoxication process but may in some cases, however, result in a product with higher toxicity. Liver microsomes contain P450 enzymes involved in detoxifying xenobiotics and these enzymes often have broad and overlapping substrate specificities whereas those P450 enzymes involved in steroid biosynthesis in the adrenal glands are far more selective in their choice of substrate.

Mechanisms of Cytochrome P450 Catalyzed Reactions

The reactions catalyzed by the cytochrome P450 enzymes require a transfer of electrons from NAD(P)H either via cytochrome P450 reductase in the endoplasmic reticulum, or via ferredoxin reductase in the mitochondria. The electron is subsequently transferred to the cytochrome P450
which results in reductive activation of molecular oxygen — one oxygen atom is inserted into the substrate (ROH) and the other into H2O, shown in Figure 6.

\[
2H^+ + RH + O_2 \rightarrow ROH + H_2O + 2e^-
\]

Figure 6. Reactions mediated by the P450 enzymes involve the conversion of an organic substrate (RH), in the presence of oxygen, to a mono-oxygenated metabolite (ROH) and water.

The reducing equivalents required for the reaction to proceed are supplied by NADPH and less often by NADH. Substrate binding causes a conformational change in the P450 enzymes which triggers the interaction of the P450 enzymes with their redox partners [Lewis, 1996 & 2001]. The reducing equivalents are transferred in two stages from NAD(P)H via one or 2 redox partners. The spin state of cytochrome P450 does not dramatically affect electron transfer from NADPH via the reductase but substrate is required for efficient electron transfer (Kominami and Takemori, 1982; Ruckpaul and Rein, 1990). The cytochrome P450 enzymes are broadly classified in two classes based on the electron transfer system they employ.

Figure 7. Schematic representation of the steroidogenic electron transfer system of mitochondria (a) and microsomes (b). Oxidized adrenodoxin (Ad\(^+\)), reduced adrenodoxin (Ad\(^-\)), substrate (S) [reproduced from Takemori and Kominami, 1984].
The mitochondrial cytochrome P450 enzymes are considered as Class I P450 enzymes since they require both a FAD containing reductase, adrenodoxin reductase (ADXR), and an iron sulphur protein/ferrodoxin, adrenodoxin (ADX), to transport the reducing equivalents (Figure 7a). The ADX forms a tightly associated bimolecular complex with the ADXR. The flavin moiety is reduced by NADPH and the electron is transferred from ADXR to the oxidized ADX which, upon reduction, dissociates from ADXR. The reduced ADX subsequently associates with the substrate-bound cytochrome P450. The electron gained by the P450 enzyme is used in the hydroxylation reactions. The transfer of electrons thus involves sequential complex formations of ADXR and ADX with the cytochrome P450, with the ADX molecule in the mitochondrial electron transfer chain ‘shuttling’ the electrons between the ADXR and the cytochrome P450 enzyme [Ziegler et al., 1999]. The microsomal P450 enzymes, categorized as Class II cytochrome P450 enzymes, require cytochrome P450 reductase to transfer the reducing equivalents directly from NADPH (Figure 7b). The reductase molecule is embedded in the membrane and contains a FAD/FMN moiety. Six to eight cytochrome P450 enzymes tend to cluster around a central reductase [Nebert et al., 1982; Ziegler et al., 1999].

The reduction of dioxide in the P450 system is carefully regulated by the redox partners, the medium which would be either membrane bound or cytosolic, the nature of the active site and its degree of solvation as well as the influence of the heme-thiolate group of the enzyme. Uncoupling of the reaction occurs if this regulation is not tightly controlled, resulting in the release of reactive oxygen species [Lewis, 2001].
Figure 8. Cytochrome CYP101 catalytic cycle. The steps represented in the figure are discussed in the text.

In the first stage of the cytochrome P450 catalytic cycle [Hall, 1986; Lewis, 2001; Porter and Coon, 1991; White and Coon, 1980] the enzyme is in its resting state during which the heme iron (Fe(III)) is in a low-spin state and the sixth distal ligand is most likely water or a hydroxyl ion (Figure 8). The substrate binds to the heme opposite the fifth ligand (Step 1), prompted by the hydrophobic attraction of the substrate for the heme pocket of the enzyme. Substrate binding displaces the water distal ligand, which converts the enzyme from a low-spin (hexacoordinate) to a high-spin state (pentacoordinate) as illustrated in Figure 9 [Sligar, 1976]. The change in spin state lowers the redox potential of the heme, facilitating the initial reduction of the substrate-bound cytochrome P450 by its redox partner (Step 2). Kominami et al. (1982) concluded from their studies that the spin state does not control the electron transfer from the NADPH but did conclude that the substrate is essential for the reduction of the P450 enzyme. One electron is taken up by the iron which is therefore reduced to its ferrous form (Fe(II)) and remains in the high-spin state. The loss of the sixth coordinate allows oxygen to bind to the cytochrome P450
enzyme by heme ligation (Step 3). Once the dioxygen molecule has bound, the ferrous iron is converted to a low-spin state. Electron transfer from iron to oxygen occurs, resulting in the formation of a ferric-superoxide species. The second electron is subsequently donated from the cytochrome P450 redox partner, activating the oxygen, followed by an internal rearrangement of the electrons restoring the iron to its ferric form (Step 4). The species formed, \( \text{Fe}^{3+}-\text{O}_2^- \), is highly reactive and may associate with 2 protons in the environment of the active site to form hydrogen peroxide. In the monooxygenase reaction, two oxygen atoms are cleaved resulting in one oxygen atom being incorporated into the substrate, the other into a molecule of water with the subsequent release of water (Step 5). The oxygen insertion into the substrate is believed to involve hydrogen abstraction from the substrate (Step 6) and then insertion of the resulting hydroxyl into the substrate (Step 7) [Hall, 1986; Lewis, 2001].

![Hexacoordinate, low spin](image1.png)

![Pentacoordinate, high spin](image2.png)

**Figure 9. Illustration of the spin state of cytochrome P450 [reproduced from Hall, 1986].**

**Catalytic Properties of Cytochrome P450 enzymes**

The cytochrome P450 active site contains a protoporphyrin IX moiety which is located in a hydrophobic cleft of the apoprotein (Figure10). The heme binds to a central metal ion which is penta- or hexacoordinate. Four of the coordinates are occupied by the planar porphyrin ring, bound by hydrophobic forces. The fifth ligand is the thiolate anion of a cystein residue while the sixth ligand is either occupied by water or, when the iron is reduced, the sixth coordinate is the site of dioxygen activation [Hall, 1986; Porter and Coon, 1991; Segall, 1997; White and Coon, 1980].
Cytochrome P450 enzymes may be characterized using UV/visible spectrophotometry, identifying different intermediates in the catalytic cycle. In the catalytic cycle of P450cam (CYP101), the substrate free enzyme exhibits an absorption maximum at 417-420 nm which is associated with the low spin state. The binding of the substrate brings about a change in the spin state to a high spin state which exhibits an absorption maximum at 391 nm. Once reduced, this complex has an absorption maximum at 408 nm and upon binding molecular oxygen the absorption maximum shifts to 418 nm. The difference in the spectrum obtained is due to the energy level transitions in the heme locus itself, which is influenced by the nature of the heme ligand, the environment the heme moiety is in and the nature of the bound substrate or inhibitor [Kominami and Takemori, 1982; Lewis, 1996]. The low and high spin states are attributed to the characteristics of the central ferric iron (Fe$^{3+}$). In the low spin state ($S = \frac{1}{2}$) the five 3d electrons are maximally paired while during the high spin state ($S = \frac{5}{2}$) the five 3d electrons are maximally unpaired [Segall, 1997]. Substrates for the P450 enzymes thus induce difference spectra when bound to the enzyme (Figure 11); however, compounds that are not considered substrates for the enzymes are also capable of inducing difference spectra. Three types of difference spectra have been characterized for Fe(III) P450, depending on the type of ligand bound in the active site: type I and II and reverse type I (or modified type II) [Lewis, 1996 & 2001].
Figure 11. Type I (dashed line) and type II (solid line) difference spectra following the addition of a type I (5 mM hexobarbital) and Type II substrate (18 mM aniline) to cytochrome P450 enzymes in the liver microsomes (5 mg/ml protein) [reproduced from Omura and Sato, 1978].

A type I difference spectrum exhibits an absorption minimum at approximately 420 nm and an absorption maximum between 385-390 nm. This reaction graphically interprets the interaction of the P450 enzyme with its substrate. Since low-spin P450 enzymes absorb at 416-420 nm, cytochromes P450 in high spin state absorb between 385-394 nm, the type I substrate shifts the Fe$^{3+}$ spin equilibrium from a low-spin to a high-spin state (Figure 10) by displacement of the iron from the plane of the ring toward the thiolate sulphur [Hall, 1987; Lewis, 1996 & 2001; Sligar, 1976]. Most cytochrome P450 substrates do induce the high-spin state of the enzyme and therefore exhibit a type I binding spectrum. This change arises as a result of displacement of the water molecule from the sixth position which causes the iron to shift out of the planar porphyrin and toward the fifth or thiolate ligand. The binding of substrates that induce a type I spectrum is illustrated in Figure 12. The percentages of the spin states can be calculated from the difference spectra.
Figure 12. An example of a substrate-bound system, camphor-bound CYP101. The binding of the substrate displaces the water molecule. The oxygen atoms are shown in red, nitrogen in light blue, iron in dark blue, sulphur in yellow and carbon atoms are shown in grey as bonds [reproduced from Segall 1997]

A type II difference spectra is characterized by an absorption minimum between 390-405 nm and an absorption maximum between 425 and 435 nm (Figure 11). As a result there is a shift to the longer wavelengths, from a high-spin state to a low-spin state during which the iron atom shifts into the plane of the heme which favours the low-spin state. The low spin form possesses a smaller ionic radius compared to the high spin form. The substrates that fall in the type II category tend to be cytochrome P450 inhibitors, inhibiting by binding directly to the Fe$^{3+}$, replacing the water molecule as illustrated in Figure 13. Most of these substrates contain atoms with non-bonded electrons, which are able to bind to the heme locus.
Figure 13. An example of an inhibitor-bound system, metyrapone-bound CYP101. The inhibitor binds directly to the heme $\text{Fe}^{3+}$, replacing the water molecule as the sixth axial ligand. The oxygen atoms are shown in red, nitrogen in light blue, iron in dark blue, sulphur in yellow and carbon atoms are shown in grey as bonds [reproduced from Segall, 1997].

A reverse type I difference spectrum earned this title because this spectrum is characteristically a ‘mirror image’ of the type I binding spectrum. It is also known as the modified type II spectrum as it closely resembles the type II binding spectrum. An absorption maximum between 409-445 nm and an absorption minimum between 365-410 nm is observed in this difference spectrum. These substrates are not thought to ligate to the heme but rather displace the distal ligand and bind elsewhere in the heme pocket [Lewis, 1996 & 2001]. These reverse type I substrates usually constitute relatively hydrophobic molecules, so that water is displaced by some form of lipophyllic interaction within the heme environment during substrate binding.

The reduced form of cytochrome P450, iron (II) P450 also has its own characteristic UV absorption spectrum, particularly in the presence of carbon monoxide. The wavelength of the Soret absorption maximum is 450 nm, hence the name, cytochrome P450 [Omura and Sato, 1964 & 1978]. Changes of the Soret peak are dependent on the environment of the heme which means
that the active site of a P450 enzyme will influence the electronic transition associated with the band around 450 nm. This method is very effective in identifying and quantifying active P450 enzymes.

Most cytochromes P450 enzymes display simple Michaelis-Menton saturation kinetics and competitive inhibition characteristics between substrates, but some isozymes display non-hyperbolic kinetics which is carefully reviewed by Hlavica and Lewis (2001). An allosteric effect may be responsible for the sigmoidal velocity versus substrate concentration curves. This can occur when either, the second substrate molecule binds with greater affinity than the first, or product formation is faster when both substrates are bound than when only one is bound to the enzyme. Atkins et al. (2002) investigated the toxilogical advantage for non-hyperbolic kinetics in the hepatic cytochrome P450 catalysis and they concluded that allostery can provide a detoxification advantage at low to intermediate substrate concentrations and that at high concentrations the allostery has neither a positive nor negative effect.

2.2.2 Mitochondrial cytochrome P450 enzymes

The cytochrome P450 enzymes, CYP11A1, CYP11B1 and CYP11B2, are embedded in the inner mitochondrial membrane of the adrenal cortex and use the mitochondrial electron transport system, containing flavoprotein and ferredoxin (adrenodoxin reductase and adrenodoxin, respectively). Some of the enzymes catalyze a single reaction while others catalyze a complex series of reactions [Payne and Hales, 2004; Ruckpaul and Rein, 1990].

Plasma cholesterol is the initial substrate for steroid hormone biosynthesis. Cholesterol is also synthesized from acetate in adrenal cells but is utilized to a lesser degree in steroid biosynthesis. Plasma cholesterol circulates as lipoproteins and comprises 80 % of the circulating lipoproteins. Cholesterol enters the adrenal cells by receptor mediated endocytosis as plasma cholesterylsters bound to low density lipoproteins (LDL) and is stored as lipid droplets. ACTH stimulates adrenal steroidogenesis since it upregulates LDL receptor expression in adrenocortical cells [Thomson, 2003]. Cholesterol bound to high density lipoproteins (HDL) destined for the liver is secreted into the bile or converted into bile salts. The catalytic activity of cholesterol esterase is
stimulated by ACTH, which liberates cholesterol from its esters to enter the mitochondria. Cholesterol is subsequently delivered to the inner mitochondrial membrane by the function of StAR, a short-lived mitochondrial import factor and not by means of passive diffusion. The translation of StAR is induced by heightened levels of cAMP and PKA which occurs upon ACTH receptor activation.

CYP11A1, which is associated with the inner mitochondrial membrane on the matrix side, catalyses the first step in steroid hormone biosynthesis. The side chain cleavage of cholesterol to form pregnenolone (PREG) requires three sequential hydroxylation reactions by CYP11A and requires 3 molecules of oxygen and 3 molecules of NADPH for each reaction (Figure 14). The first hydroxylation takes place at C22, followed by a C20 hydroxylation to produce 20a,22-dihydroxycholesterol. The intermediate is then cleaved between C20 and C22 to produce the C21 steroid, PREG and isocaproaldehyde. The CYP11A1 enzyme is expressed in all three adrenal cortex zones, in the ovaries, testis and placenta of humans [Payne and Hales, 2004]. This enzyme has also been detected in the central and peripheral nervous system of mice as well as in the rat brain [Compagnone et al., 1995; King et al., 2004; MacKenzie et al., 2002].

The second group of mitochondrial cytochrome P450 enzymes includes, CYP11B1 and CYP11B2, which catalyze the final reactions in adrenal steroidogenesis. These enzymes are expressed in the adrenal cortex and low levels of expression have been identified in areas of the brain [MacKenzie et al., 2002; Yu et al., 2002; Mellon and Griffin, 2002]. CYP11B1 is mainly expressed in the zona fasciculata and zona reticularis but has been observed in the mitochondrial zona glomerulosa, whereas, CYP11B2 is expressed exclusively in the zona glomerulosa of the adrenal cortex [Payne and Hales, 2004]. The CYP11B1 enzyme catalyses 11β-hydroxylation (Figure 15A) of 11-deoxycorticosterone and 11-deoxycortisol (preferred substrate) to form...
corticosterone and cortisol, respectively [Payne and Hales, 2004; Ruckpaul and Rein, 1990]. CYP11B2 catalyses three sequential reactions converting 11-deoxycorticosterone to the mineralocorticoid, aldosterone. The first reaction catalyzed is the 11β hydroxylation of 11β-deoxycorticosterone, followed by the hydroxylation of C18 and finally an oxidation of the C18 hydroxyl group to form aldosterone (Figure 15B). The intermediates are formed without being released from the active site. Each of the reactions requires one molecule of oxygen as well as one molecule of NADPH. Unlike CYP11B2, CYP11B1 is not capable of 18-hydroxylations and therefore plays a more important role in the biosynthesis of the glucocorticoids namely, corticosterone and cortisol [Kawamoto et al., 1992]. Cortisol secretion usually exceeds (100-1000 fold) that of aldosterone. This phenomenon is not only due to the transcriptional regulation of these enzymes but rather due to the differences in catalytic activities of these two enzymes [Lisurek and Bernhardt, 2004].

Figure 15. Reactions catalyzed by CYP11B1 (A) and CYP11B2 (b) [reproduced from Payne and Hales, 2004].
2.2.3 Microsomal cytochrome P450 enzymes

Both CYP17 and CYP21 are present in the endoplasmic reticulum and catalyze the hydroxylation at the C17 and C21 position in the adrenal microsomes. Electrons for both CYP17 and CYP21 are supplied by NADPH-cytochrome P450 reductase.

PREG is hydroxylated by the CYP17 enzyme to form 17α-hydroxypregnenolone (17-OH-PREG). CYP17 subsequently catalyses the 17,20-lyase reaction of the intermediate to produce DHEA (Figure 16). Similarly, progesterone (PROG) is also 17α-hydroxylated, to form 17α-hydroxyprogesterone (17-OH-PROG) which can be converted to androstenedione (A4) in some species [Ruckpaul and Rein, 1990]. In humans PROG also converted to 16-OH-PROG. CYP17 is therefore capable of catalyzing both the 17α-hydroxylation and the 17,20-lyase reaction of PREG and PROG. CYP17 is at a branch point in the production of glucocorticoids and adrenal androgens (DHEA and A4). In the production of glucocorticoids, the 17α-hydroxysteroid intermediates are further hydroxylated by CYP21 (discussed below) whereas in the production of adrenal androgens the 17α-hydroxysteroid intermediates are lysed by CYP17 to form the adrenal androgens [Ruckpaul and Rein, 1990]. The CYP17 enzyme is expressed in the adrenal cortex (only the zona fasciculata and zona reticularis), in the testis and in the ovaries [Machino et al., 1969]. Mutations in the CYP17 gene lead to a reduction of both reactions catalyzed by CYP17, resulting in a reduced production of the androgens, and in the abnormal development of secondary sex characteristics.

Figure 16. Reactions catalyzed by CYP17 [reproduced from Payne and Hales, 2004]
CYP17 is a complex enzyme with the hydroxylase activity of the enzyme being similar across species but marked differences reported with regards to the lyase activity of the enzyme. In addition, the preferred substrate for CYP17 differs across species — human CYP17 more readily converts the $\Delta^5$ steroids while rodent CYP17 prefers the $\Delta^4$ steroids as substrate [Brock and Waterman, 1999; Nakajin and Hall, 1981]. The hydroxylase and lyase activity is also tissue specific. Brock and Waterman (1999) indicated that the presence of cytochrome b5 stimulates lyase activity in bovine and human adrenals, while Swart et al. (2003) concluded that a lack of lyase activity in the ovine adrenal is as a result of the absence or presence of a modulating condition or substance (phosphorylation or membrane environment) other than cytochrome b5.

The second cytochrome P450 present in the endoplasmic reticulum of adrenocortical cells is CYP21, which catalyses the 21-hydroxylation of PROG and 17-OH-PROG to DOC and deoxycortisol, respectively (Figure 17). The CYP21 enzyme is expressed in all three zones of the adrenal cortex [Payne and Hales, 2004] and CYP21 activity [Mellon and Miller, 1989] and expression [Beyenburg et al., 2001] has also been demonstrated in the human brain.

![Figure 17. Reactions catalyzed by CYP21](reproduced from Payne and Hales, 2004).

3β-Hydroxysteroid dehydrogenase (3βHSD) belongs to the short-chain alcohol dehydrogenase family [Penning, 1997]. Two distinct isoforms of this enzyme have been identified in humans namely, 3βHSD type I (expressed in the placenta, skin and breast tissue) and 3βHSD type II (expressed in the adrenal gland, ovary and testis). This enzyme is responsible for the conversion
of PREG to PROG, 17-OH-PREG to 17-OH-PROG and DHEA to A4 [Payne and Hales, 2004; Ruckpaul and Rein, 1990]. The Michaelis-Menten constant (Km) of 3βHSD is lower for DHEA than for 17-OH-PREG or PREG [Conley and Bird, 1997]. Two sequential reactions convert the Δ⁵-3β-hydroxysteroids to Δ⁴-3-ketosteroids (Figure 18). The first step involves the 3β hydroxyl group followed by the isomerization Δ⁵-3-ketosteroids to form the Δ⁴-3-ketosteroids. The dehydrogenation reaction requires NAD⁺ which, when reduced, activates the isomerization reaction. The Δ⁴-3-ketosteroids are formed without the release of an intermediate or coenzyme.

Figure 18. Reactions catalyzed by 3βHSD [reproduced from Payne and Hales, 2004].

2.2.4 Inhibition of cytochrome P450 enzymes

The P450 enzymes may be inhibited by a variety of molecules in a variety of ways: heme ligation, hemeadduct formation, competition inhibition, formation of a reactive intermediate or a combination of the aforementioned [Halpert, 1995; Lewis 1996 & 2001]. In addition, cytochrome P450 inhibitors may be selective in that they inhibit a single P450 enzyme while others are non-selective and inhibit many P450 enzymes. Carbon monoxide, nitric oxide and cyanide inhibit cytochrome P450 enzymes non-selectively and readily bind irreversibly to the heme iron. Selective cytochrome P450 enzyme inhibitors usually inhibit by competitive inhibition and are often type II ligands binding reversibly to the heme iron.
Organic ligands such as quinoline, furan, benzimidazole, pyridine and triazole, contain sterically unhindered nitrogen or oxygen atoms with free valence electrons which act as inhibitors by binding reversibly to the heme iron [Testa and Jenner, 1981]. Some cytochrome P450 enzyme inhibitors interact with the heme moiety of the enzyme as a result of an enzyme-mediated activation — the metabolic intermediate formed by the enzyme complexes with the heme [Murray and Reidy, 1990]. This type of inhibition is referred to as mechanism based inhibition. A selective inhibitor for CYP21, 21,21-dichloroprogesterone, is an example of such a mechanism-based inhibition.

Metal ions also act as cytochrome P450 inhibitors and appear to inhibit by competing with the iron for the heme porphyrin. Competition would be greatest with metal ions that have the closest ionic charge, ionic radius and redox potential to iron, of which manganese (Mn$^{2+}$), nickel (Ni$^{2+}$), cobalt (Co$^{2+}$) and cadmium (Cd$^{2+}$) are examples.

Various inhibitors of the cytochrome P450 enzymes are of pharmacological importance and are used in treating diseases of the adrenal gland. Increased levels of cortisol and aldosterone contribute to severe diseases such as Cushing’s syndrome, metabolic syndrome, hypertension and increased mortality in congestive heart failure [Bureik et al., 2004; Muller-Vieira et al., 2005]. Metyrapone, a known inhibitor of CYP11B1, which catalyses cortisol biosynthesis, lowers cortisol levels and is most commonly used in the treatment of Cushing’s syndrome [Verhelst et al., 1991]. Aminoglutamamide, a more toxic drug, is prescribed together with metyrapone and inhibits earlier cytochrome P450 enzymes in the steroidogenic pathway, thus not only inhibiting cortisol biosynthesis [Child et al., 1976]. Trilostane inhibits 3βHSD and is used in patients with adrenal adenomas [Semple et al., 1983]. Trilostane inhibition is not used in treatment of Cushing’s syndrome because inhibition is overcome by the rise in ACTH. Ketoconazole is widely used as an anti-fungal agent, which can cause abnormal liver function. Ketoconazole also contains the potential to lower cortisol levels by blocking various steroidogenic cytochrome P450 enzymes [Contreras et al., 1985; McCance et al., 1987]. Mitotane is an adrenolytic drug used in the management of adrenal carcinoma and is taken up by both normal and malignant adrenal tissue [Nieman, 2002]. Thus, finding selective inhibitors for the steroidogenic enzymes which catalyze the reactions involved in steroid biosynthesis,
lowering the levels of cortisol and aldosterone, would be useful for the treatment of diseases associated with their chronic elevation.

2.3 Summary

Psychological stress results in the activation of the HPA-axis, leading to elevated levels of plasma glucocorticoids. The stress-induced rise in glucocorticoid levels does not protect us from the source of stress but rather from the body’s reaction to stress, minimizing an overreaction that might threaten homeostasis. The HPA-axis is a complex system that is intricately regulated by neural and endocrine systems. Glucocorticoids, cortisol and corticosterone regulate the HPA-axis by negative feedback. The negative feedback is assumed to be sufficiently delayed to allow the appropriate defence mechanism to be activated. Plasma cortisol concentrations are regulated by the concentrations of the plasma steroid binding proteins and their free versus bound ratios. DHEA is also hyper secreted in chronic stress situations, antagonizing the effects of cortisol. Cortisol biosynthesis and secretion into the systemic circulation is regulated by ACTH. Regulation of on a transcriptional level is associated with effects relating to long term exposure to stress while acute effects influence the secretion of the hormones. Cortisol is synthesized in the adrenal gland from cholesterol by the cytochrome P450 enzymes. Chronic elevations of ACTH increase the synthesis of the cytochrome P450 enzymes. The activity of the cytochrome P450 enzymes is regulated by the availability of: cholesterol, the initial substrate for steroid hormone biosynthesis; NADPH, as an electron source; and the reductases, for the transport of electrons from NADPH to the P450 enzyme.

In chronic stress situations, the negative feedback regulation of the HPA-axis is overridden, leading to permanently elevated levels of basal plasma cortisol concentrations. Prolonged exposure to elevated concentrations of cortisol are associated with a number of ailments and in general renders one more susceptible to disease [Chrousos 1999]. Adverse effects associated with elevated cortisol levels may be alleviated by inhibition of the cytochrome P450 enzymes responsible for cortisol biosynthesis. Although the P450 enzymes are highly specific for their natural steroid substrates, inhibitors of the expressed P450 enzymes are usually not specific for single P450 enzymes but have inhibitory activity on all the P450 enzymes of the steroidogenic
pathway. Compounds similar in structure to the natural steroids may inhibit the catalytic activity of cytochrome P450 enzymes binding in the heme pocket of the enzymes. In addition, the cytochrome P450 enzymes can be inhibited by influencing other role players required for enzymes catalysis, such as the phospholipid environment and electron transport systems.
Chapter Three

*Sutherlandia frutescens*

Natural products represent an enormous reservoir of molecular diversity to drug discovery and development. Approximately 50% of drugs in clinical use comprise natural plant products and their derivatives and higher plants represent no less than 25% thereof [Van Wyk and Wink, 2004]. It took many centuries, through a process of trial and error, to develop the profound knowledge of traditional medicine. Since the functions of the body are currently better understood we are able to appreciate the potential of plants in treating complicated health conditions by acting as multifunctional chemical entities. Although natural products are normally taken in their crude form as teas (infusions), sophisticated phytomedicines have been standardized and extracts of plants have been formulated and undergone rigorous testing. The use of natural products and phytomedicines has become a more popular option than medicinal products derived from synthetic chemicals. The emphasis at present is therefore to support serious medical claims made by traditional medicines with clinical studies, establishing the safety and efficacy of these practices.

South Africa boasts a rich plant and cultural diversity and a large number of plants are used for medicinal purposes, one of which is *Sutherlandia frutescens*. It is estimated that there are approximately 3000 South African plant species used for medicinal purposes [Light et al., 2005; Van Wyk et al., 1997]. Since South Africa is a developing third world country, herbal medicines form the backbone of rural healthcare, not only for economical reasons but also for cultural reasons.

3.1 Botanical nomenclature, description and distribution

The *Sutherlandia* (L.) R.Br genus belongs to the Fabaceae family and is commonly known as Cancer bush. The Fabaceae (pea & bean or pod-bearing family) is the second largest flowering plant family which contains 600 genera and 12 000 species, distributed throughout the world.
This family of plants is represented in Southern Africa by 134 genera and more than 1300 species. *Sutherlandia* comprises 6 taxa, all endemic to Southern Africa [Moshe et al., 1998] and closely related to the *Lessertia* genus, sometimes considered a part thereof. Analysis of the gene product of 32 enzyme coding loci, performed by Moshe et al (1998), revealed genetic variation in 18 loci. Large overlaps were found in cluster analysis of genetic distance data indicating that members of the *Sutherlandia* taxa are not easily genetically distinguishable. The genus *Sutherlandia* was named after James Sutherland, the first Superintendent of the Edinburgh Botanic Garden and the species *frutescens* means bushy in Latin. Common names for *S. frutescens* are balloon pea, Umnwele (Xhosa and Zulu), kankerbos (Afrikaans), Insiswa, Mukakan and Phetola. *S. frutescens* is often confused with its other closely related species, *Sutherlandia montana* (mountain cancer bush), *Sutherlandia microphylla* and *Sutherlandia tomentosa*. Lack of agreement between morphological and allozyme patterns within the genus as well as low allozyme differentiation between populations and taxa may be a result of the breeding mechanism [Moshe et al., 1998].

The malachite sunbird (*Nectarinia famosa*) pollinates the *Sutherlandia* taxa [Arroyo, 1981]. The birds’ ability to travel long distances makes it possible to transfer genetic material from geographically isolated populations and consequently result in the recombination of alleles between taxa. It has been suggested that the flowers may become cleistogamous, enabling self-pollination, resulting in a population becoming larger till pollinators are attracted [Moshe et al., 1998]. The high level of integration and recombination among the taxa does not fully support the idea that the genus comprises 6 taxa. Furthermore, Moshe et al (1998) found it impossible to differentiate between the taxa due to a lack in unique alleles. The significant characteristics distinguishing the different taxa are habitat, orientation of fruit stipe, shape and pubescence of the leaflets and the shape of the pods [Moshe et al., 1998]. Results obtained from a combination of characteristics have, to date, not conclusively established the genus to which the shrub belongs. Since it was found that the *Sutherlandia* taxa have very low genetic differentiation, the South African genus may belong to one variable taxon, *S. frutescens*.

*S. frutescens* is a small attractive shrub and the lively colours of the shrub appeal to many. The *Sutherlandia* taxa were in fact grown as ornamentals as early as 1683 in England and some species are still popular garden varieties. The perennial, soft wooded shrub grows up to 2 meters
in height. The leaves which are bitter in taste, are divided into smaller leaflets (4-10 mm) which may be slightly to densely hairy giving the plant a silvery appearance [Van Wyk et al., 1997]. The shrub produces large (35 mm long), bright red flowers in spring to mid-summer (September-December), followed by almost transparent bladder-like papery pods. The six species identified are, however, hard to distinguish from each other.

*Sutherlandia* genus is distributed mainly in the arid regions of southern Africa, in Western Cape and Karoo regions of South Africa as illustrated in Figure 1 [Fernandes et al., 2004; Van Wyk et al., 2000]. The *S. frutescens* species is restricted to southern Africa and occurs in Namibia, Botswana, and South Africa [Van Wyk et al., 1997]. *S. frutescens* populations usually comprise a relatively small number of plants in an area of less than 500m². These populations are widely distributed and show remarkable regional variation. Raw materials for medicinal purposes were usually wild harvested with selected chemotypes cultivated on a commercial scale. *S. frutescens* is a hardy, fast growing plant which tolerates all soil types. Although *S. frutescens* occurs in summer and winter rainfall regions, it thrives in full sun and is drought resistant. Seeds are usually sown in autumn or spring in well drained soil. Germination usually occurs 2-3 weeks after planting.

![Figure 1. Distribution of *S. frutescens* in South Africa [Van Wyk et al., 1997]](image)

### 3.2. Phytomedicines and traditional remedies

Phytomedicines are derived from medicinal plants and affect various biochemical pathways either directly or indirectly to restore physiological equilibrium and balance. The
bioactive compounds of this category of medicinal plant products have in most cases been identified and quantified, allowing the marketing of a standardised final product. A few examples of natural medicines include St John's wort (*Hypericum perforatum*), *Echinacea* (*Echinacea purpurea*) and *Ginkgo biloba*. Traditional remedies on the other hand lack standardised levels of bioactive compounds. However, the popularity of herbs and botanicals is supported by the demand, as is reflected in the United States consumer market which comprises half the total market for supplements. These supplements include homeopathic products, vitamins, minerals and sport supplements [Van Wyk and Wink, 2004; Eisenberg *et al.*, 1998]. In 1998, the value of trade in ethnomedicinal plants in KwaZulu-Natal alone was estimated to be worth R60 million [Mulholland and Drewes, 2004]. A survey performed by the World Health Organization indicated that 70-80% of the world's population relies on non-conventional medicine (mainly herbal sources) as their primary healthcare [Akerele, 1993]. It is thought that orthodox medicines are considered to be an allopathic form of treatment while alternative medicines, herbal or traditional remedies, involve a holistic approach for the body's self-regeneration [Chan 2003].

African traditional medicines are considered to be the oldest and most diverse of all medical systems but are, unfortunately, also the most poorly recorded [Van Wyk and Wink, 2004]. African herbal remedies stem from the Khoi-San and Nama people of Southern Africa who themselves are considered the most ancient culture in Africa. In South Africa, traditional medicine is more accessible than Western medicine for the majority of the population and it is estimated that 60% of the population consult one of 200,000 traditional healers [Van Wyk *et al.*, 1997]. Medicinal plant tonics are used to maintain and support general and physical health in healthy individuals while, during illness, they assist in restoring health by possibly stimulating the immune function. Medicinal plant tonics enhance metabolism and improve digestion through stimulation of the gall-bladder. Liver and kidney function is also stimulated to aid secretion and excretion of waste products [Van Wyk *et al.*, 1997; Van Wyk and Wink, 2004]. Traditional healers in South Africa are most commonly known as, amongst others, 'inyanga' and 'isangoma' which refers exclusively to herbalists and diviners respectively. This distinction, however, has become blurred, with some healers practicing both arts. In South Africa, the claimed effects of only a small number of approximately 3000 medicinal plants have been scientifically validated. Although the change in the socio-political climate over the past 10 years has increased consumer
awareness, data with regards to the safety, efficacy, and bioactivity of medicinal plants remains limited. Funding supporting research of African traditional medicines stimulated ethnobotany and ethnopharmacology research to shift towards the integration of traditional herbal medicine into primary healthcare [Light et al., 2005].

Members of the Fabaceae family are associated with strong antibacterial activity (Erythrina lysistemon Hutch), antifungal activity (Glycyrrhiza glabra L.), antimalarial activity (Caesalpinia volkensii Harms) and antioxidant potential [Van Wyk and Wink, 2004]. S. frutescens is one of the few South African medicinal plants marketed internationally and is currently considered the most profound adaptogenic tonic in South Africa.

3.2.1 Preparation and dosage

Traditional healers take many factors into account when harvesting medicinal plants — the regional climatic conditions, aspect of mountain slope, type and moistness of the soil and time of day. These factors have been identified by healers to influence the potency of medicinal plants. Various processing procedures may also affect the chemistry of the medicinal plants. Some plants are used in the fresh state while many are dried in the sun or shade or may be cut into slices to dry. Plant material may be stored as is or reduced to a fine powder and stored in paper bags, newspapers, glass jars or tin cans [Van Wyk et al., 1997].

African, Western, as well as Chinese herbal remedies are usually specific mixtures or formulas of up to 20 different types of herbs. The recipes of these traditional medicines are contained in ancient compendia. It is important to understand the nature of the active compound(s) since the use of alcoholic extracts (tinctures) for example, may result in ineffective treatment or harmful side effects compared to the use of traditional water extracts. Not only is the manner of administration important for the effectiveness of the compound(s) but so is the correct dosage [Van Wyk and Wink, 2004]. Herbal drugs or botanicals usually have a wide therapeutic window in that the effective dose differs substantially from the toxic dose. However, as with every rule there is always the exception and qualified individuals should therefore be responsible for
prescribing the remedies. Pure compounds on the other hand have a much smaller window and the desirable dose is easily exceeded to reach toxic levels [Van Wyk and Wink, 2004].

The biologically active compounds of plants are often found in different parts of the plant, while one plant part may be toxic, another may be harmless. In phytotherapy it is either the whole plant or a specified part of the plant that is used. Bark is used frequently in phytotherapy since it often contains high concentrations of active components, for example, quinine bark (*Cichona* species: *Chinae cortex*) and willow bark (*Salix* species: *Salicis cortex*) [Van Wyk and Wink, 2004]. Many other specific parts of plants containing bioactive compounds are used in herbal remedies — the flower, wood, leaf, seed, fruit, resin, gum, bulb, tuber or root. There are also a number of forms in which herbal medicines can be administered — teas, decoctions, macerations, infusions, juices, syrups, oils, pills, lozenges, capsules, tablets and even spirits or essences. The polarity, stability and other chemical characteristics of the active ingredient(s) in medicinal remedies and the ease with which the active ingredient(s) enter the human target cells, determines the route of administration. Different herbal medicines have been identified to be active only when administered orally, nasally, topically, rectally, by smoking or steaming, bathing and subcutaneous or intramuscular injection [Van Wyk and Wink, 2004]. In addition, more volatile compounds may be effective when inhaled but inactive when taken orally.

The leaves of *S. frutescens* are mainly used in traditional remedies but all above ground parts of the plant are usually included [Van Wyk et al., 1997]. Strong decoctions or alcoholic tinctures are administered and are sometimes mixed with other ingredients. Decoction refers to a preparation made by adding cold water to the required amount of herb which is boiled for 5 to 10 min and strained. An alcoholic tincture contains 70-30 % alcohol in which the plant material is boiled for a specific time and strained. These traditions stem from the Khoi and Nama people who used decoctions externally to wash wounds and internally for fevers and other ailments [Van Wyk et al., 1997]. Traditionally, 1-2 g of *S. frutescens* dry herb was administered as a tea or decoction [Van Wyk and Wink, 2004]. The recommended dosage of *S. frutescens* for humans is 9mg/kg body weight per day [Seier et al., 2002]. This recommended dosage is based on the
tablets of the dry herb currently on the market\textsuperscript{1}, which recommend two tablets a day each containing 300 mg of dried plant material.

\subsection*{3.2.2 Medicinal potential of traditional remedies - efficacy and safety issues}

Efficacy of a traditional medicine is evident at different levels with information being passed down from generation to generation, as well as from practitioners making clinical observations. Pharmacological studies further demonstrate activity \textit{in vitro}, \textit{in vivo}, \textit{ex vivo} and observational studies, which include large groups of people, may provide information showing statistically significant benefits. Clinical studies would, however, provide the most significant and convincing scientific method for proving safety and efficacy of traditional medicines [Van Wyk and Wink, 2004]. Clinical trials involve four phases which are usually monitored on a yearly basis. After preclinical trials, 30-40 healthy patients are examined to determine the best and safest dose as well as the best form of administration. The next phase requires the participation of 100-1000+ people to determine if the drug is effective and for further evaluation of its safety. The final phase also involves a large group of people to test the drug against a current standard treatment. A successful trial would show a statistically significant difference between the treated and control groups.

Standardized extracts of traditional medicines are established by testing the biological activity of different extracts of the medicinal plant. After safety and toxicity studies of the standardized extract have been completed, industrial production can begin. Clinical tests are subsequently carried out which may result in the extracts approval for drug development. The approval of specific chemical compounds isolated from medicinal plants is, however, more complex and time consuming. The processes required for the approval of standardized extracts and pure active compounds is summarized in Figure 2. Specific chemical compounds may only be approved for industrial production after structure-activity relationship studies (SARs), toxicity and safety studies, clinical tests and an appropriate pharmaceutical formulation has been established [Pieters

\textsuperscript{1} www.bioharmony.co.za
www.health-connection.co.za
and Vlietinck, 2005]. Some of the general requirements for a bioassay used in the screening of plant extracts include: validity, lack of ambiguity, accuracy, reproducibility, simplicity and economic viability. It is therefore evident that the investigation of medicinal plants is a multidisciplinary process. Since the approval of standardized extracts of medicinal plants is more rapid and economically viable, it is the starting point for a successful pharmaceutical industry in developing countries.

Verpoorte et al (2005) suggest that efficacy studies of traditional medicine requires a holistic approach which includes mental, social and physiological factors as apposed to the single compound single target approach. The holistic or in vivo approach for testing medicinal plants can be achieved by either clinical trials or animal studies. Quantitative analyses of all metabolites in the test organism may determine the holistic effects of herbal remedies, an ultimate though somewhat ambitious goal [Patwardhan, 2005; Verpoorte et al., 2005]. Methods most commonly

Figure 2. Processes for studying efficacy of standardized medicinal plant extracts as well as active compounds for their approval as drugs [Pieters and Vlietinck, 2005]. SAR-studies refer to the structure-activity relationship studies.
used to identify and quantify the various metabolites include chromatography based methods (HPLC, GC), molecular weight based methods (mass spectrometry) and physical characteristic based methods (NMR spectrometry) [Verpoorte et al., 2005]. These analytical procedures are not only important methods in analyzing changes in metabolites in the test organism but also in analyzing the metabolites present in the plant itself. It is important that active compounds are isolated and identified, not necessarily for drug development but to understand the efficacy of medicinal plants in human health care and determine the bioavailability of the bioactive compounds in a biological system.

Poisoning from traditional medicines can be a consequence of misidentification, incorrect administration, dosage or preparation of the plant material, due mostly to self-administration [Barnes and Prasain, 2005; Fennell et al., 2005]. Johannesburg’s forensic database for 1991 to 1995 revealed that 43 % of the poisoning cases were attributed to traditional plant medicines [Stewart and Steenkamp, 1999]. When compared to accidental deaths from inappropriate use of conventional medicines, toxicity of traditional medicines is rare. Herbal remedies may be contaminated with excessive or banned pesticides, microbial contaminants, heavy metals, chemical toxins or even the presence of orthodox or conventional drugs [Chan, 2003; De Smet, 2002; Marcus and Grollman, 2002]. The source of these contaminants can be associated with the cultivation, drying, storage or processing of the plant material. Furthermore, the quality of herbal medicines may be compromised by a plant’s popularity or scarcity, resulting in the trade of poor quality plants or in the deliberate substitution with other plant material for economical reasons. The substitution of other or poor quality plants may, however, be an error in incorrect identification. Incorrect possessing procedures may result in toxicity as the processing and preserving procedures normally reduce toxic components in crude extracts and may also enhance the activity of the active compounds. It is advisable that the bioactive plant extracts be tested for toxic effects since the difference between the levels of toxicity toward the parasite versus toxicity towards the host may be minimal. Analytical methodologies to assess contaminants present in herbal remedies are carefully assessed in the review article by Chan (2003).

Misidentification of medicinal herbs may inevitably result in misadministration of the herbs. The identification of the different medicinal plants and their extracts may be addressed by using chromatographic techniques. Springfield et al (2005) investigated HPLC fingerprinting in the
identification and quality assessment of South African herbal medicines. In cases where botanical identification was impossible, HPLC with diode array detection (DAD) was used to provide a qualitative profile or chemical fingerprint of the compounds present in the crude drugs or their extracts. HPLC-DAD is a sensitive, rapid and economical technique which can be implemented in developing countries with relative ease to promote the rational use of medicinal plants. The qualitative identification of chemical compounds by HPLC is achieved by comparison of retention data. Springfield et al. (2005) compared chromatograms of 3 different collections of *Chironia baccifera* which were qualitatively sufficiently similar. Since plant material contains highly variable concentrations of active compounds these differences in concentrations can be clearly observed on the chromatograms. HPLC methods of identification may be used to regulate concentrations of active compounds in medicinal plants, once a therapeutic concentration range has been established [Van Wyk and Wink, 2004]. Interestingly, the different analytical procedures used to identify and quantify some of the compounds present in the herbal remedies have identified surprisingly few compounds for conventional drug use. This suggests synergistic actions of other compounds in the herbal remedy which are either chemically unknown or have not been isolated and identified in the herbal mixture.

The absence of regulation together with the increased popularity of medicinal plants in South Africa has brought the danger of misadministration and possible genotoxic effects associated with prolonged use to the fore. Without regulation, contaminants may be present and remain unidentified in herbal remedies possibly leading to adverse side effects [Chan, 2003; De Smet, 2002; Marcus and Grollman, 2002]. The presence of contaminants in medicinal remedies is being acknowledged since 50 South African plants were recently screened for genotoxic or mutagenic effects [Verschaeve et al., 2004]. Screening has, however, identified compounds with the following properties — antibacterial, antifungal, anthelmintic, anti-amoebic, antischistosomal, antimalarial, anti-inflammatory and antioxidant potential, neurotropic and psychotropic activity [Fennel et al., 2004]. Thus, the investigation of traditional medicines is not only invaluable in ensuring the safety of medicinal plants for their continued use but also for the discovery of novel drugs. The administration of medicinal plants should be controlled by standard operating or regulatory procedures to reduce risks and to support the belief that traditional medicines may be safer than synthetic medicines. The regulation of traditional medicines should not only occur at the level of product development but practitioners should be
registered and strict standards put into place to avoid prescriptions of potentially lethal doses [De Smet, 2002; Marcus and Groliman, 2002]. Furthermore, regulation and quality control would ensure the correct identification of the medicinal plants, therapeutic concentrations of active ingredient, purity and hygiene, as well as skilled practitioners. The conservation status of medicinal plant resources requires attention since over-collecting may lead to the extinction of medicinal plant species. These aspects together with the effect of cultivation and post-harvest storage on the biological activity of medicinal plants are not addressed fully as they do not fall within the scope of this study [Light et al., 2005; Fennel et al., 2004].

3.3. Bioactive compounds

Bioactive compounds identified in plants are usually secondary metabolites or natural products which are low molecular weight compounds that do not play a role in primary plant metabolism. Although the plant often uses secondary metabolites as a defence mechanism or to attract pollinators, these compounds are not essential for the survival of the plant. There are 3 major groups of secondary metabolites which include the nitrogen-containing substances (includes the non-protein amino acids), the terpenes (includes saponins) and the phenolics.

These secondary plant metabolites must ultimately interact with a molecular target in the human body for the compounds to be therapeutically effective. Since extracts of medicinal plants contain a variety of chemical entities, the plant is able to affect more than one molecular target, which increases the chances of efficacy, restoring health or relieving symptoms of physiological disturbances. Allopathic or conventional medicines, however, use a single compound to treat disorders. Some active compounds have been isolated from medicinal plants and are used in treatment as a single chemical entity — atrophine, reserpine morphine, quinidine, digoxin or vinblastine [Van Wyk and Wink, 2004]. In contrast, a single medicinal plant may contain many active compounds which would include a bitter substance for stimulation of digestion, phenolic compounds which act as anti-oxidants and venotonics, diuretic substances to enhance the elimination of waste products and toxins, anti-bacterial and antifungal tannins, anti-inflammatory compounds as well as alkaloids to enhance moods to give a sense of well being.
Although no alkaloids have been identified in *S. frutescens*, a small number of saponins and other bioactive compounds have been characterized. One of these compounds L-canavanine, a non-protein-α-amino acid, acts as an arginine mimic to reduce the uptake of essential amino acids from the intestine thus disturbing protein biosynthesis. L-canavanine has also been documented to exhibit anticancer and antiviral activity, as well as the ability to inhibit nitric oxide synthase [Riganti *et al.*, 2003; ABD EL-Gawad and Khalifa, 2001]. There is approximately 30-40 mg of L-canavanine per dry gram of the *S. frutescens* leaf [Gericke *et al.*, 2001]. Tablets of *Sutherlandia* from Phyto Nova, analysed by Tai *et al* (2004), contained 3 mg of canavanine per gram of tablet [Tai *et al.*, 2004]. *S. frutescens* is also rich in gamma-aminobutyric acid (GABA) (14 mg per gram dry leaf, 0.4 mg/g Phyto Nova tablet). GABA is an inhibitory neurotransmitter that could account for the therapeutic use of the *S. frutescens* and *S. microphylla* for anxiety and stress symptoms [Van Wyk *et al.*, 2000]. The presence of these compounds in extracts of *Sutherlandia* tablets, shown in Figure 3, were demonstrated by Tai *et al.* (2004). The leaves also contain pinitol [Van Wyk *et al.*, 1997 Van Wyk and Wink, 2004] which is a known antidiabetic agent and has potential in treating muscle wasting in cancer and AIDS patients [Van Wyk and Wink, 2004]. The structures of these compounds are illustrated in Figure 4.

Saponins are an important group of secondary plant metabolites which include triterpenoids, glycosylated triterpenoids, glycosylated steroids and steroidal alcohols [Haralampidis *et al.*, 2002]. The triterpenoids, present in many plant extracts including *S. frutescens*, have been associated with bitter tonic effects, corticomimetic activity, anti-inflammatory, anti-ulcer, antinociceptive and anti-tumoral properties [Inoue *et al.*, 1990; Navarette *et al.*, 2002; Fernandes *et al.*, 2003; Haridas *et al.*, 2004]. The corticomimetic activity may be attributed to the structural similarity to the endogenous hormones (glucocorticoids), which would account for the anti-inflammatory effects of *S. frutescens* treatment. The triterpenoid present in *S. frutescens* is a triterpenoid glucoside known as Su1. In addition, some flavonoid compounds have been identified in the leaves of *S. frutescens*. In general, flavonoids have been associated with scavenging of free radicals, anti-inflammatory activity and the suppression of nitric oxide production [Saija *et al.*, 1995; Solimon and Mazzio, 1998; Fushiya *et al.*, 1999; ABD EL-Gawad and Khalifa, 2001]. The structure of a flavonoid and triterpenoid are illustrated in Figure 5 to demonstrate their structural similarity to the steroid hormones.
Figure 3. LC/MS APCI chromatogram of amino acids and active compounds present in *S. Frutescens* PhytoNova tablets extracts. The confirmation of pinitol was done using negative ion electrospray LC/MS [Tai et al., 2004].

![Chemical structures of L-canavanine, Pinitol, and GABA](image)

Figure 4. Active compounds present in *S. frutescens* [Van Wyk, 1997].

![Chemical structures of quercetin (flavonoid) and triterpenoid saponin](image)

Figure 5. The typical structure of a flavonoid, quercetin, found in apples, onions and teas [ABD EL-Gawad and Khalifa, 2001] and a triterpenoid saponin isolated from the fruits of *Ternstroemia japonica* [Shin et al., 2003].
3.4. Pharmacological effects of *S. frutescens*.

While herbal remedies are preferred for the treatment of chronic self-terminating conditions, conventional medicine is used to treat acute or serious illnesses. As previously mentioned, *S. frutescens* is a bitter tonic which has been used to treat stomach problems and improve quality of life [Van Wyk 2004]. Traditionally, *S. frutescens* has also been used to treat colds, influenza, chicken-pox, diabetes, varicose veins, piles, inflammation, liver problems, backache, rheumatism, stress and anxiety [Van Wyk et al., 1997; Van Wyk and Wink, 2004; Neuwinger, 2000]. Furthermore, the plant has been used in the prevention and treatment of cancer, hence its common name, Cancer bush [Moshe et al., 1998]. Cancer patients as well as AIDS and TB patients tend to lose weight and ‘waste’ away. Anecdotal reports suggests that *S. frutescens* dramatically improves the appetite and wasted patients gain weight and have increased energy levels which enhance their sense of well-being [Morris, 2002]. In addition, the extracts have been successfully used topically in the treatment of burns, wounds, inflammation and other skin conditions [Van Wyk et al., 1997]. Traditionally no distinction is made between the use of *S. frutescens* and *S. microphylla* for medicinal purposes [Van Wyk et al., 2000]. Although very little scientific evidence is available on the medicinal properties of *S. frutescens*, tablets pressed from the whole dried plant material have been manufactured and are marketed by Phyto Nova, Cape Town, as a direct result of the many positive anecdotal reports. However, in the past two years there has been an increasing interest in the use of *S. frutescens* as a medicinal remedy, most specifically in the treatment of cancer and AIDS. Many research groups are currently investigating the bioactivity of *S. frutescens* to determine the mechanism of action by which the therapeutic effects are exerted and thus establishing a scientific basis for these effects. A toxicology study has been carried out in vervet monkeys and showed that administration of *S. frutescens* at nine times the recommended dose, 381 mg/kg body weight, had no toxic or side effects, with regard to the haematological and biochemical parameters measured [Seier et al., 2002].

Anti-inflammatory properties of medicinal plants can be partly attributed, to their anti-oxidant potential. Hydrogen peroxide and superoxide radicals are phagocyte derived reactive oxygen species (ROS) that are responsible for the pathogenesis of various inflammatory conditions. A study by Fernandes et al. (2004) tested the effect of a hot water extract of *S. frutescens* on
luminol and lucigenin enhanced chemiluminescence by L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-stimulated neutrophils and investigated the superoxide and hydrogen peroxide scavenging properties in cell free systems. Results indicated that *S. frutescens* had potent antioxidant potential by decreasing superoxide and hydrogen peroxide induced chemiluminescence at concentrations as low as 10 µg/ml and 0.62 µg/ml, respectively. In addition, they found that the extract had no adverse side effects on cell viability at concentrations as high as 40 µg/ml. Since *S. frutescens* possesses significant ROS scavenging properties in cell free and in stimulated neutrophil systems, the plant may therefore be an effective immunomodulator in the treatment of diseases associated with an overproduction of ROS by human phagocytes. The anti-oxidant activity may be attributed to the phenolic compounds present in the extract, such as tannins and flavonoids but further analyses are required to identify the relevant bioactive compound(s) [Fernandes *et al.*, 2004]. In contrast to the findings of Fernandes *et al.* (2004), Tai *et al.* (2004) analysed the antioxidant potential of ethanolic extracts on nitric oxide production and reported no significant inhibition.

Although there is progress in the development of anticancer therapies, the incidence of cancer is still on the rise worldwide. Chemoprevention is an approach widely used in cancer treatment and refers to the use of a non-toxic substance either of natural or of synthetic origin, to block, reverse or retard the process of carcinogenesis. Investigations into the chemopreventive properties of *S. frutescens* were carried out by Kundu *et al.* (2004) who investigated the influence of methanolic extracts of dried leaves of *S. frutescens* on the activity of cyclooxygenase (COX) 2. COX 2 catalyses prostaglandin biosynthesis and is induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). They reported that the topical application of a methanolic extract of *S. frutescens* (100-200 µg) significantly decreased the TPA- induced COX-2 expression (26 and 48 % inhibition at doses of 100 and 200 µg respectively) in mouse skin. Evidence suggests that the targeted inhibition of inappropriately elevated expression or activity of COX 2 is desirable not only for alleviating inflammation, observed in numerous malignancies, but also for preventing cancer [Surh *et al.*, 2001]. In determining possible mechanisms for the observed inhibition, they also found that *S. frutescens* was capable of inhibiting transcription factors such as the activator protein-1 (AP-1) and the cyclic AMP response element binding (CREB) protein. In addition, they observed inhibition of the catalytic activity, but not the phosphorylation, of extracellular signal-regulated protein kinase (ERK), an upstream mitogen-activated protein kinase (MAPK).
Although the inhibition is observed it is uncertain which active compounds are contributing toward the inhibitory effect. It is possible that L-canavanine may be responsible for the benefits reported by cancer patients since the compound exhibits anti-tumourigenic properties [Yang et al., 2002; Van Wyk et al., 1997]. Furthermore, Tai et al. (2004) demonstrated antiproliferative effects of an ethanolic extract of S. frutescens in various cancer cell lines. The antiproliferative activity differed for each cell line and flow cytometry analysis indicated that the cell lines were arrested at different phases of the cell cycle, suggesting more than one active compound. L-canavanine, at high doses, was found to block all DNA synthesis, providing support for its role in contributing to the anticancer properties of S. frutescens, but the concentrations of L-canavanine used to achieve this result are much higher than concentrations present in the plant extract [Tai et al., 2004]. More recently Chinkwo (2005) studied the anti-cancer effects of S. frutescens by determining whether extracts were capable of inducing apoptosis in cultured carcinoma cells. Apoptosis is programmed cell death, which if manipulated, can be used as yet another therapeutic approach to cancer therapy. Apoptotic cell death is a physiological mechanism that eliminates unwanted cells by triggering the cell’s intrinsic suicide programme. Apoptosis is characterized by morphological changes such as membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation, followed by the engulfment of the resulting cell debris by the neighbouring cells [Christop, 2003]. Chinkwo (2005) used several apoptotic tests to validate these properties of S. frutescens in vitro. The results showed that S. frutescens exhibited apoptotic activity in three different cell lines by displaying morphological disintegration and other changes in the cellular membrane viz. the flip-flop translocation of membrane phospholipids indicative of apoptosis. The plant material investigated was collected from three different regions (Kirstenbosch, Bloemfontein and the Free state) and interestingly, all had different apoptotic activity, with the extracts from the Western Cape plant specimens showing the greatest apoptotic activity. This observation emphasized the importance of soil composition and other environmental factors on plant metabolites. Although the mechanism of apoptosis is not fully understood, Chinkwo (2005) proposed that S. frutescens activates the central death caspase 3 that is regulated by the apoptosome complex.

With over 40 million people worldwide infected with HIV, the World Health Organization has embarked on a plan to have 3 million people taking antiretroviral therapy by 2005 [Duval, 2001; Morris, 2001; Morris, 2002]. In response, the South African Department of Health has accredited
27 facilities to provide AIDS care, including nutritional and micronutrient supplementation as well as complementary and traditional medicines to delay the progression of the disease. Products from medicinal plants have been reported to inhibit nearly all the stages of the viral life cycle of HIV in vitro — sulphated polysaccharides (inhibit viral attachment) and calanolode A (inhibits HIV reverse transcriptase) and other compounds with unknown modes of action [Yang et al., 2001]. Anecdotal reports from doctors and health care workers suggest that *S. frutescens* treatment in HIV-infected individuals results in weight gain, improved CD4 counts, decreased viral loads, improved appetites and general mood improvements [Chaffey and Stokes, 2002]. The nutritional status of the HIV-infected individual can seriously influence the efficacy of anti-retroviral and *S. frutescens* treatments. For example, *S. frutescens* contains L-canavanine which is an arginine mimic which could enfold into the individuals proteins causing the immune system to turn on itself, leading to serious diseased states. Thus a balanced diet, providing adequate concentrations of arginine to out compete L-canavanine, is required to eliminate the toxicity of L-canavanine. Anecdotal evidence suggesting *S. frutescens* may have antiviral properties prompted Harnett et al. (2005) to investigate the possible inhibitory effects of extracts on HIV. The inhibition of the glycohydrolase enzyme was investigated since the inhibition of this enzyme was found to decrease the infectivity of the HIV virion [Collins et al., 1997; Harnett et al., 2005]. Results indicated that *S. frutescens* extracts contain inhibitory compounds active against HIV target enzymes which include the glycohydrolase enzyme and HIV reverse transcriptase (inhibition >50%), showing a potential mechanistic action of the plant in aiding HIV-positive patients [Harnett et al., 2005]. Interestingly, inhibition of the reverse transcription assay was reduced in the presence of BSA, suggesting that tannins may be responsible for the observed effects. However, *S. frutescens* extracts still retained ~30% inhibition after the addition of BSA, showing thus that the anti-HIV effects observed may be attributed to other compounds.

Many antiretroviral medications, which are predominantly metabolized through the cytochrome P450 3A4 (CYP3A4) oxidative metabolic pathway, are HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors. HIV protease inhibitors are also substrates for drug transporters like P-glycoprotein. The potential of *S. frutescens* to cause drug interactions with anti-retroviral metabolizing mechanisms was investigated in vitro, testing the inhibitory effects of the herb on CYP3A4 activity and P-glycoprotein expression. *S. frutescens* showed inhibition
of both and in so doing highlighted the importance of \textit{in vivo} investigations into the clinical ramifications of the use of herbs or traditional medicine together with anti-retroviral therapy [Mills \textit{et al.}, 2005a; Mills \textit{et al.}, 2005b].

As previously mentioned, the \textit{S. frutescens} plant has also been used to treat stress. In fact, the plant’s Zulu name, unwele, meaning hair, refers to the fact that administration of the plant stops people from pulling out their hair in distress. The success of traditional medicine in treating mental illness is often attributed to the placebo effect rather than active compounds producing the physiological response. However, active entities have been identified for psychological treatment. The alkaloid compound isolated from \textit{Sceletium tortuosum} (L.) N.E. Br. (Mesembryanthemaeae) patented in 1997 (World Patent 9746234, 1997 Gericke and Van Wyk) has been shown to play a role in the selective re-uptake of serotonin. It is administered in the therapeutic management of depression [Fennell \textit{et al.}, 2004]. Chronic stress results in heightened activation of the HPA-axis, resulting in chronically elevated cortisol levels which are associated with a number of maladies as discussed in the previous chapter. Both cancer and HIV/AIDS are also associated with an increased response to chronic stress. In a rat model of chronic immobilization stress, Smith and Myburgh (2004) studied the effect of \textit{S. frutescens} treatment on selected serum hormone and cytokine levels. \textit{S. frutescens} treated rats had decreased corticosterone levels in response to chronic intermittent stress when compared to the control. It is thought that the decrease in glucocorticoid levels may be responsible for the reported improvement of quality of life and the decrease in muscle wasting of cancer and HIV/AIDS patients. Possible target tissues for the anti-stress function of \textit{S. frutescens} include the hippocampus, where stress perception may be downregulated; the hypothalamus and pituitary, where activation of the HPA-axis may be downregulated; or the adrenal gland where cortisol biosynthesis may be downregulated.

\subsection*{3.5 Summary}

\textit{S. frutescens}, an attractive indigenous garden shrub, cultivated as early as the seventeen century, has traditionally been used to treat a wide variety of ailments which presently also include HIV and stress. The importance of phytomedicines, medicinal plant products and herbal remedies in
the treatment of diseases is reflected in consumer market [Van Wyk and Wink, 2004; Eisenberg et al., 1998]. In South Africa, traditional remedies are more accessible to the greater population than conventional health care. The use of medicinal plants and herbal remedies in primary health care should therefore be regulated and standardized, ensuring that this tradition remains safe and economically viable. Imperative too to their prolonged therapeutic use is the need for scientific validation — evidence supporting therapeutic claims of traditional remedies. Investigations into the bioactivity of *S. frutescens* have shown anti-oxidant, anti-inflammatory, anti-stress, antiproliferative and apoptotic activity, as well as conflicting reports in some instances [Fernandes et al., 2004; Tai et al., 2004; Kundu et al., 2004; Chinkwo, 2005; Smith and Myburgh, 2004]. Recent analysis of *S. frutescens* extracts identified bioactive compounds which include L-canavanine, pinitol, GABA, triterpenoids and flavonoids and these have been linked to specific therapeutic applications [Riganti et al., 2003; ABD EL-Gawad and Khalifa, 2001; Van Wyk et al., 2000; Van Wyk and Wink, 2004; Inoue et al., 1990; Navarette et al., 2002; Fernandes et al., 2003; Haridas et al., 2004; Saija et al., 1995; Soliman and Mazzio, 1998; Fushiya et al., 1999]. In HIV treatment, it was reported that although *S. frutescens* was capable of decreasing the virulence of the HIV virion, it also interacted with CYP 3A4 and other enzymes catalysing the metabolism of antiretroviral drugs [Mills et al., 2005a; Mills et al., 2005b; Harnett et al., 2005]. Research is therefore required to determine the physiological consequences of treatment with *S. frutescens* when used in conjunction with antiretrovirals.

It is evident that therapeutic claims of *S. frutescens* are being validated by scientific evidence on many fronts. In the next chapter evidence is presented supporting the potential stress relieving properties of *S. frutescens*. The alleviation of negative effects associated with chronically elevated cortisol plasma levels, a phenomenon experienced by both cancer and HIV/AIDS patients as well as chronically stressed individuals, may be linked to the inhibitory effects of *S. frutescens* on adrenal cytochrome P450 enzymes. Investigations were carried out to determine the influence of *S. frutescens* extracts and of *S. frutescens* in tablet form on adrenal steroidogenesis. These results together with investigations into the bioactivity of compounds present in *S. frutescens* viz. pinitol, L-canavanine, GABA, triterpenoids and flavonoids, are presented and discussed in chapter 4.
Chapter Four

Inhibition of steroidogenic P450 enzymes by S. frutescens

4.1 Introduction

*Sutherlandia frutescens* has been used traditionally as a herbal remedy to treat the symptoms of stress as well as many other ailments [Van Wyk and Wink, 2004]. The stress relieving properties of the plant have been linked to its ability to influence plasma glucocorticoid concentrations [Smith and Myburgh, 2004]. It was shown that *S. frutescens* extracts not only decreased corticosterone levels in rats subjected to chronic intermittent immobilisation stress but also increased basal corticosterone levels in non-stressed rats. *S. frutescens* may therefore exert its stress relieving properties by influencing the HPA-axis acting as an adaptogen as well as by down-regulating glucocorticoid production. As mentioned previously, the HPA-axis is stimulated in response to stress which results in an increase in the concentration of circulating glucocorticoids. The glucocorticoids released form part of a negative feedback mechanism to regulate the activity of the HPA-axis and endocrine system. This regulatory feedback mechanism is overridden in chronic psychological stress and diseases such as HIV/AIDS and cancer, resulting in long-term exposure to elevated plasma glucocorticoid levels [Huizenga *et al.*, 1998]. Negative side effects resulting from chronically elevated glucocorticoid levels [Chrousos, 1998] may be alleviated by treatments which suppress glucocorticoid biosynthesis. Treatment would therefore require the inhibition, or interference with the activity of the cytochrome P450 enzymes which catalyse the biosynthesis of the glucocorticoids, cortisol and corticosterone. The aim was therefore to determine if *S. frutescens* was able to influence the steroidogenic cytochrome P450 enzymes, thereby demonstrating a possible mechanism for reducing glucocorticoids levels.

The bioactivity of *S. frutescens* was assayed by investigating the effect of aqueous and methanol plant extracts, single bioactive compounds and commercially available tablets on adrenal cytochrome P450 enzymes. Although the therapeutic effects of a medicinal plant are thought to be contributed to by compounds exerting synergistic effects, single compounds were also investigated to determine if the effects could be attributed to any of the compounds tested.
Compounds which were assayed for bioactivity include gamma-aminobutyric acid (GABA), pinitol, L-canavanine, flavonoids and triterpenoids. GABA has been associated with mood elevating properties [Van Wyk et al., 2000]; pinitol has anti-inflammatory properties [Van Wyk and Wink, 2004] and L-canavanine has anti-viral and anti-cancer properties [ABD EL-Gawad and Khalifa, 2002; Riganti et al., 2003].

Bioactivity assays were carried out based on the unique spectroscopic properties of the cytochrome P450 enzymes and on the conversion of specific substrates by these enzymes. Two spectral assays, assay A and B were conducted. In assay A the ability of *S. frutescens* to induce an inhibitor-induced difference spectrum was investigated. In assay B the effect of *S. frutescens* on a substrate-induced difference spectrum was investigated. Since the ability of *S. frutescens* to influence the conversion of cortisol precursors would serve as a measure of biological activity, substrate conversion assays were carried out in the presence of *S. frutescens*. Two assays were conducted in which the effect of *S. frutescens* on the conversion of PREG and PROG was determined. Assay A was conducted in ovine adrenocortical microsomes and assay B in COS1 cells, a non-steroidogenic mammalian cell line. Expressing CYP17 and CYP21 in COS1 cells allowed the determination of effects of *S. frutescens* on the catalytic activity of the individual enzymes. As previously mentioned, CYP17 is a complex enzyme, at a branch point in the production of glucocorticoids and adrenal androgens. Compounds affecting the catalytic activity of CYP17 and CYP21 would not only influence the biosynthesis of cortisol but also the precursor intermediates.

### 4.2 Materials and Methods

#### 4.2.1 Materials

*Sutherlandia* specimens were collected from the Western Cape region of South Africa, and identified as *Sutherlandia frutescens* subspecies *microphylla*³. Plant material, leaves and stems, were dried at ambient temperature in the shade for at least 5 days. *Sutherlandia* tablets were purchased from Bioharmony (Phyto Nova) and Health Foods, South Africa. Sheep adrenals were

---

³ Kind gift from B-E van Wyk (Dept. Botany, University of Johannesburg, South Africa)
collected from the Maitland Abattoir in the Western Cape. GABA, pinitol and L-canavanine were purchased from Sigma Chemical Co (South Africa).

PEG 8000 was purchased from Sigma-Aldrich Chemie (Germany). PROG, PREG, DOC, isocitrate and isocitrate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NADH and NADPH were purchased from Boehringer Mannheim GmbH (Germany). Radioactive tritiated steroids \[^3H\]PROG and \[^3H\]PREG were purchased from PerkinElmer Life Sciences (Boston, MA, USA). COS I cells were purchased from ATCC, USA. Dulbecco’s Modified Eagles Medium (DMEM) was purchased from Sigma–Aldrich and Dulbecco’s phosphate buffered saline was purchased from Gibco, Invitrogen Corp. (Canada). Fetal calf serum and DMSO were purchased from Highveld Biological (South Africa). Penicillin-streptomycin and Tripsin-EDTA were purchased from Invitrogen Corp. (Canada).

Spectra were recorded on a Varian Cary 219 spectrophotometer. Chromatography was performed on a Waters (Milford, MA, USA) high performance liquid chromatograph coupled to a Waters 700 satellite WISP™ automatic injector and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL) as well as a Waters absorbance detector (254 nm). Scintillation fluid was purchased from Beckman Coulter Inc. (USA). All other chemicals were of reagent grade and purchased from either Sigma Chemical Co. or Merck Laboratory Supplies (South Africa).

### 4.2.2 Preparation of *S. frutescens* extracts

Dried, ground *S. frutescens* plant material, 18.6 g, was placed in a glass soxlet extractor fitted with a double wall condenser [Swart et al., 1986, 1993; de Villiers, 1990]. The extractor was fitted to a round bottom flask and the plant material was extracted with 250 ml methanol for ~8 hrs. The extract was dried at reduced pressure on a rotary evaporator at room temperature, yielding 2.87 g dried plant material. The dried methanol extract was re-dissolved in 35ml de-ionized water. An aqueous extraction was prepared by boiling dried plant material, 2.16 g, in de-ionized water, 275 ml, for 30 min. The aqueous extract was lyophilized, yielding 0.624 g plant material, and re-dissolved in 13 ml de-ionized water. The extracts were centrifuged at 6000 g for 5 min and the supernatants were stored at 4°C. The final concentration of the methanol and aqueous extracts was 82 mg/ml and 48 mg/ml, respectively.
GABA, pinitol and L-canavanine were dissolved in de-ionized water to a concentration of 1 mg/ml. The triterpenoid and flavonoid fractions of *S. frutescens* were also prepared. The lyophilized flavonoid fraction was re-dissolved in de-ionized water and the lyophilized triterpenoid fraction was re-dissolved in de-ionized water containing 0.1 % ethanol. Stock solutions of 1 mg/ml and 5 mg/ml were prepared and stored at 4°C.

*Sutherlandia* tablets, 700 mg containing 300 mg dried leaf powder, were crushed to a fine powder and dissolved in 5ml de-ionized water. The samples were incubated at 37°C for 1 h and centrifuged at 3000 x g for 5 min. The supernatants were stored at 4°C till further use.

### 4.2.3 Preparation of adrenal mitochondria and microsomes

The mitochondrial and microsomal fractions were prepared as previously described by Ming-Xue Yang & Arthur L. Cederbaum (1994). Fresh ovine adrenals were used for the isolation of partially purified cytochrome P450 enzymes and all procedures were carried out in a cold room at 4°C. The adrenals were decapsulated and 25 g adrenal cortex tissue was homogenized in 75 ml 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.25 M sucrose, first in a Hamilton Beech blender and subsequently with a Potter-Elvejhem glass homogenizer. The homogenate was centrifuged at 1000 x g for 20 min. The pellet was discarded and the supernatant was centrifuged at 12000 x g for 15 min to sediment the mitochondria. The mitochondrial pellet was washed by resuspension in 200 ml 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA and 1 % BSA and centrifuged at 12000 x g for 15 min. The washing step was repeated twice as previously described. The washed mitochondrial pellet was lyophilized overnight and the resulting ovine adrenal mitochondrial powder, 0.46 g, was stored at -18°C.

PEG 8000 (50 % w/v) was slowly added to the supernatant containing the microsomal fraction to a final concentration of 8.5 %. The mixture was stirred for 10 min at 4°C and centrifuged at

---

*Donated by Prof. B-E van Wyk, Dept. Botany, University of Johannesburg, South Africa.*
13000 x g for 20 min after which the supernatant was discarded. The pellet was subsequently homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl and 1 mM EDTA. PEG (50 % w/v) was added to the suspension to a final concentration of 8.5 %, stirred for 10 min at 4°C and centrifuged at 13 000 x g for 20 min. After repeating this step once more, the microsomal pellet was resuspended in 80 ml 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, and stored in 5 ml aliquots at -80°C.

4.2.4 Determination of cytochrome P450 concentration

The cytochrome P450 content of the adrenal microsomes and mitochondria was determined by the carbon monoxide method described by Omura & Sato (1964).

The mitochondrial powder (2 mg/ml) was sonicated in 0.1 M phosphate buffer (pH 7.4) containing 10 % ethylene glycol, in an ice slurry for 5 min with one minute intervals. The sonicate was subsequently saturated with carbon monoxide, divided into 2 optically matched cuvettes and a baseline recorded between 400 and 500 nm. Sodium dithionate (1-2 µg) was added to the sample cuvette and the spectrum recorded. The spectra were monitored for ~10 min until the spectrum was completely developed.

The microsomal suspension was diluted (1:3) in 0.1 M phosphate buffer and the carbon monoxide induced difference spectrum of the microsomal preparation was carried out in the same manner. A molar extinction coefficient (ε) of 91 cm⁻¹mM⁻¹, as reported by Omura and Sato (1964), was used to determine the cytochrome P450 concentration (c) from the equation below.

\[ \Delta A = \varepsilon c l \]

\( \Delta A \) is the change in absorbance, \( c \) is the concentration of the solution and \( l \) is the path length through the solution (1 cm) [Wilson and Walker, 2000]. The protein concentration of the microsomal and mitochondrial preparations was determined using the Pierce BCA Protein Assay Kit (Perstorp Life Sciences Company, IL, USA).
4.2.5 Spectral binding assays

Assay A: Inhibitor-induced difference spectra

The assays were carried out to determine whether the test components were able to bind to adrenal cytochrome P450 enzymes using microsomal preparations. The microsomes were diluted in 0.1 M phosphate buffer, (pH 7.4) containing 10 % ethylene glycol to a cytochrome P450 concentration of 0.6 μM (7.9 mg protein). The assays were carried out at room temperature in a final reaction volume of 1 ml using four optically matched cuvettes, two reference cuvettes and two sample cuvettes, in tandem. The test component (methanol and aqueous extracts (to a final concentration of 4.1 mg/ml and 2.4 mg/ml respectively), tablet extracts (0.3 mg/ml) and bioactive compounds (0.05 mg/ml)) was added (50 ul) to one reference and one sample cuvette which contained 0.2 M phosphate buffer. The remaining two cuvettes contained the enzyme preparation. A baseline was recorded between 360 nm and 500 nm. Prior to recording the binding spectrum, the test component in the sample cuvette was replaced with 0.2 M phosphate buffer. Test component was subsequently added to the enzyme preparation in the sample cuvette and an equal volume of buffer was added to the enzyme preparation in the reference cuvette. Spectra were recorded over a period of 30 min until the spectrum was completely developed.

Assay B: Inhibition of steroid-induced difference spectra

These assays were done to determine whether the test components were able to inhibit the binding of endogenous steroids to the mitochondrial and microsomal P450 enzymes. Reactions were carried out at room temperature in a final reaction volume of 1 ml [Swart et al., 1993; 2003]. The cytochrome P450 concentration was 0.78 μM and 0.35 μM in the mitochondrial and microsomal preparations, respectively. The inhibitory effects of the test components on the steroid-induced difference spectra, were determined by adding 5 μl - 150 μl (to a final concentration of 0.24 mg/ml – 12.3 mg/ml) of the extracts and compounds (0.05 mg/ml – 1.5 mg/ml) to the enzyme preparations. Equal volumes were subsequently divided into 2 optically

5 The tandem configuration for reference and sample cuvettes was required to eliminate the absorbance the test components exhibited in the Soret range (personal communication P Swart)
matched cuvettes and a baseline was recorded between 360 and 500 nm. The steroid substrates, PREG, PROG and DOC, dissolved in ethanol, were added to the sample cuvette to a final concentration of 3.2 μM. An equal volume of ethanol was added to the reference cuvette. The cuvettes were inverted gently and the spectra recorded.

The substrate-induced difference spectrum of CYP17 and CYP21 was determined with PREG and PROG as substrate in the microsomal suspensions. The substrate-induced difference spectrum of CYP11B1 was determined in the same manner using DOC as a substrate in the mitochondrial suspension. These spectra were recorded using plant material (final concentrations of 0.24 mg/ml – 12.3 mg/ml) and tablet extracts (3 mg/ml), the triterpenoid (0.05 -1.5 mg/ml) and flavonoid fractions (0.05 -0.15 mg/ml), L-canavanine (0.05 – 0.15 mg/ml), GABA (0.05 – 0.15 mg/ml) and pinitol (0.05 – 0.15 mg/ml). The control substrate-induced difference spectra were recorded in the absence of the test components. Inhibition was indicated by a reduction in the amplitude of the peaks – a decrease in the absorbance maximum at 383 nm and an increase in the absorbance minimum at 416 nm. The inhibitory effect may be calculated as follows:

\[
\% \text{ inhibition} = 100 - \left( \frac{t}{c} \right) \times 100, \text{ where } t = \text{absorbance at } 390 \text{ nm minus absorbance at } 420 \text{ nm in the presence of inhibitory component and } c = \text{absorbance at } 390 \text{ nm minus abs at } 420 \text{ nm in the absence of inhibitory component.}
\]

4.2.6 Steroid conversion assays

Assay A: steroid conversion assays in adrenal microsomes

Steroid substrate conversion assays serve as a measure of biological activity and were thus conducted to determine if S. frutescens was able to influence the conversion of steroid substrates in the microsomal preparations. The metabolism of PREG and PROG was assayed in ovine adrenal microsomes as previously described [Swart et al., 1995; 2002]. The assay was carried out in a final volume of 1 ml in a shaking water bath at 37°C. Radio-labelled tritiated steroid (50000 cpm/50 μl) and steroid solution (10 μM) were pipetted onto filter paper, placed in a microcentrifuge tube and dried under nitrogen. Once the organic solvents had been evaporated, 50 mM Tris buffer (pH 7.4) containing 50 mM NaCl and 1% BSA, microsomal preparation (0.35
μM P450, 3.96 mg protein), 10 mM MgCl₂ and isocitrate (2 mg/ml) was added to the dried steroids. The mixture was preincubated for 5 min at 37°C and the reaction initiated by the addition of isocitrate dehydrogenase (0.4 U/ml) and NADPH (1 mM). The inhibitory effect S. frutescens was assayed by the addition of the test components in a 50 μl volume (50 μl buffer or solvent in the control assay) to the reaction mixture prior to the initiation of the reaction. With the addition of 50 μl of the extracts to the assay, the final concentration of the dried plant material in the presence of the methanol and aqueous extract was 4.1 mg/ml and 2.4 mg/ml, respectively. The addition of 50 μl of a 5 mg/ml stock solution of the triterpenoid fraction resulted in 0.25 mg/ml final concentration while the addition of 50 μl of S. frutescens tablet extracts represented a final concentration of 3 mg/ml dried plant material. At specific time intervals (0, 1, 2, 5 and 10 min) 50 μl aliquots were removed and pipetted into glass tubes containing a cold mixture of 5ml dichloromethane and 450 μl de-ionized water. The steroid metabolites were extracted by vortexing the mixture for 2 min and the organic and water phases separated using a bench centrifuge at medium speed. The water phase was subsequently aspirated and the dichloromethane evaporated under nitrogen. The steroid products were redissolved, in 120 μl methanol for HPLC analysis.

Assay B: steroid conversion assays in COS1 cells

Since microsomal preparations contain both CYP17 and CYP21, the influence of S. frutescens on these individual enzymes was investigated by expressing the recombinant enzymes in COS1 cells, a non-steroidogenic mammalian cell line. The lyase activity of the CYP17 enzyme is species specific and steroid conversion was thus investigated with CYP17 enzymes from different species to establish if S. frutescens had specific effects on the hydroxylase or lyase activity of the enzyme.

Maintenance of COS 1 cells

All incubations were carried out at 37°C and cells were maintained at 5 % CO₂ and 90 % humidity. All additions or removal of media were performed in sterile conditions in a laminar flow hood. A freezing vial containing 1 ml COS 1 cells was thawed in hand and resuspended in 10 ml of culture medium (DMEM, high glucose containing 0.15 % NaHCO₃, 10 % Fetal calf
serum and 1% penicillin streptomycin solution) warmed to 37°C. The suspension was plated into a 100 mm culture dish and incubated. The culture medium was replaced daily with 10 ml warmed culture media until the cells were confluent (4 days). Confluency was greater than 80%.

The cells were split into 5 x 100 mm culture dishes as follows: The medium was removed from the 100 mm culture dish and washed with 1 ml warm trypsin-EDTA medium (10% trypsin-EDTA and 10% Dulbecco’s phosphate buffered saline, without calcium and magnesium). The cells were incubated at room temperature with 1 ml trypsin-EDTA medium for 3 min before collecting the cells (> 80% confluency). The cells were subsequently resuspended in 50 ml culture media and 10 ml was plated into each of the 5 culture dishes.

Freezer stocks were prepared by collecting cells in culture media (50 ml/100 mm dish), as described above. The cell suspension was pipetted into a conical centrifuge tube and centrifuged at 500 x g for 5 min. The media was removed and the cells were resuspended in culture medium containing 10% DMSO, 3 ml/100 mm dish. Aliquots, 1 ml, were frozen at -80°C for 48 hours and subsequently transferred to liquid nitrogen and stored till further use.

**Transfection of COS 1 cells**

Cells were split one day prior to transfection, as described above. On the following day the culture media was removed and each culture dish was washed with 2 ml warmed transfection medium (DMEM, high glucose containing 0.15 NaHCO3, 1% penicillin streptomycin solution, 20 mM HEPES, pH 7.4). The cells were subsequently incubated for 1 h in 2 ml transfection media containing 0.25 mg/ml DEAE-dextran and 10 µg/ml plasmid DNA. The following plasmid constructs containing the recombinant DNA encoding the cytochrome P450 enzymes were assayed: baboon CYP17/pTarget, ovine CYP17/pCDNA 3.1, human CYP17/pCD and bovine CYP21/pCMV. The transfection media was removed and 10 ml culture media containing chloroquine, 52 µg/ml, was added to each dish and incubated for 5 h. Each culture dish was subsequently washed with 5 ml culture media and incubated with 10 ml fresh culture media. The culture media was replaced daily. Transfected cells were split into 12 well plates 48 h after transfection, as previously described. Cells from 1 x 100mm were resuspended in 12 ml culture media and 1 ml cell suspension was plated per well. Cells were incubated for 24 h before the addition of the substrate.
Substrate addition and conversion assay

The culture media was removed and the enzymatic reactions were initiated by the addition of 1 ml culture medium containing the appropriate steroid substrate (1 μM) together with tritiated steroid (100 000 – 200 000 cpm/ml). A dilution range (1-1000 X) of the *S. frutescens* extracts was made in de-ionized water and comprised 0.2 ml of the 1 ml volume of the substrate containing culture medium added to the cells and incubated for 12 - 24 h. The extracts were redissolved in de-ionized water, the influence of the presence of this volume of extract was determined by substituting 0.2 ml of the substrate containing culture medium with the de-ionized water which served as the control. In addition the following transfection controls were included to ensure that the metabolism of the steroids were the result of the presence of the expressed enzyme: a mock transfection in which no plasmid was added to the transfection media and a transfection in which cells were transfected with a vector containing no recombinant DNA.

Substrate conversion in the absence and presence of *S. frutescens* extracts was assayed by removing 0.1 ml media at over a period of 24 h and extracting steroid metabolites with 5 ml dichloromethane as previously described (section 4.2.6, Assay A). Samples were vortexed and centrifuged after which the aqueous phase was aspirated and dichloromethane phase dried under nitrogen. The dried steroid residues were subsequently redissolved in 120 μl methanol, transferred into WISP™ vials and subjected to HPLC analysis.

COS 1 cells viability assay

The viability of the COS 1 cells exposed to *Sutherlandia* extracts was assessed by monitoring the detachment of cells from tissue culture dishes after prolonged (24 h) contact with the plant extracts⁶. The experiments were conducted as follows. The methanol and aqueous extracted plant material were resuspended in water and sterile filtered (0.22 μm). COS 1 cells (26mm culture dish) were incubated with extracts (4.1 mg/50 μl methanol extract and 2.4mg/50 μl aqueous extract, respectively) for 24 hours, the media was removed and the culture dishes were washed (3 times) with PBS (1ml per well) to remove detached cells. The attached, viable cells were subsequently collected with a cell scraper and resuspended in PBS (3 ml). COS cells incubated

⁶ http://www.jingmei.com/site/site/zt/Roche/FuGENE%206%20Feature%20Presentation.pdf
without extracts served as a control and were treated in the same manner. The cells were washed by centrifugation at 500 x g for 5 min, aspirating the PBS supernatant and re-suspending the cells in 1 ml PBS. This was repeated 3 times to remove all traces of culture medium. The cell suspensions were subsequently homogenised with a mini Potter Elvejham homogeniser (1 ml) and the protein concentrations for each incubation determined using the Pierce BCA Protein Assay Kit (Perstorp Life Sciences Company, IL, USA).

4.2.7 HPLC analysis of steroid metabolites

PREG metabolites were separated using a Novapak® C8 column at a flow rate of 1 ml/min [Swart et al., 1995; 2002]. The mobile phase consisted of solvent A (50:48.5:1.5 water/acetonitrile/isopropanol) and solvent B (100 % acetonitrile). Metabolites were eluted from the column with solvent A for 5 min followed by a linear gradient from A to 100 % B in 3 min and an isocratic elution with solvent B for 3 min.

The PROG metabolites were separated on a Novapak® C18 column at a flow rate of 1 ml/min. Two mobile phases, solvent A (65:35 methanol/water) and solvent B (100 % methanol) were used to achieve the desired resolution of the steroids [Swart et al.,1995; 2002]. The metabolites were eluted from the column with solvent A for 15 min followed by a linear gradient from A to 100 % B in 5 min and an isocratic elution with solvent B for 5 min.

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

% inhibition = 100 - ((t/c) x 100), where t = % conversion or formation of steroid in the presence of the inhibitory component and c = % conversion or formation of steroid in the absence of inhibitory component.
4.2.8 Liquid chromatography-Mass spectrometry of *S. frutescens* extracts.

LC-ESMS was performed with a Micromass Quattro hyphenated quardropole time-of-flight mass spectrometer. A capillary voltage of 3.5 kV was applied with a source temperature of 100°C. The cone voltage was set at 35 V. Data acquisition was in the negative mode through the \( m/z = 100 - 1999 \) scan range. A 10 \( \mu l \) injection of the aqueous samples was separated on a Waters Alliance HPLC fitted with a Luna Gemini C18 (5 \( \mu m \), 30 x 2 mm) column. Two solvents were used to obtain the desired separation; solvent A, de-ionized water containing 0.1 % formic acid and Solvent B, 100 % acetonitrile. Separation was obtained with the gradients shown in Table 1. A flow rate of 1 ml/min was applied with a split of 4:1 directed to the mass spectrometer and the rest to a photo diode array detector (scanning from 220 nm – 550 nm).

Table 1. Gradient table for HPLC separation of *S. frutescens* extracts

<table>
<thead>
<tr>
<th>Time</th>
<th>Solvent A</th>
<th>Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>98.0</td>
<td>2.0</td>
</tr>
<tr>
<td>30.00</td>
<td>0.0</td>
<td>100.00</td>
</tr>
<tr>
<td>35.00</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>35.01</td>
<td>98.0</td>
<td>2.0</td>
</tr>
<tr>
<td>45.00</td>
<td>98.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

4.2.9 Statistical analysis

Results are expressed as the mean with error bars representing the standard errors of the means (SEM). The Student’s paired t-test was used to compare the means of two groups using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com".
4.3 Results

The biological activity of *S. frutescens* extracts, single compounds and tablets were investigated by determining their ability to bind P450 enzymes, with the subsequent induction of a type II or a reverse type I spectrum, as well as the influence of the test components on the binding of substrates to the P450 enzymes. As discussed previously, the three types of spectra, type I, type II and reverse type I (modified type II), shown in Figure 1, may be identified by changes in the UV spectrum. Ligand free cytochrome P450 exhibits an absorption maximum at ~420 nm since the ferric iron is in a low spin state. A typical type I binding spectra, achieved upon binding to the substrate, exhibits a reduced absorption at 420 nm and a corresponding increased absorption maximum at 390 nm. The binding of substrate to the enzyme is characterised by a change from the low spin to the high spin state of the ferric iron. Compounds which interfere with the binding of the substrate or prohibit the substrate from binding will result in an increase in the absorption at 420 nm and a decrease in the absorption at 390 nm. Inhibitors binding directly to the ferric iron, replacing the water molecule as the sixth axial ligand, will induce a type II spectrum. This spectrum is characterised by an increase in the absorption between 425 nm and 435 nm and a decrease in the 390 nm maximum, due to an increase in the low spin state of the ferric iron. The reverse type I spectrum is characterised by an increase in the 420 nm band and a decrease in absorbance at 390 nm. It is possible that these changes may result from the ligand binding to the enzyme but does not, however, result from direct binding to the ferric iron.

![Figure 1](http://scholar.sun.ac.za)

Figure 1. Typical example of difference spectra obtained by the addition of a substrate or an inhibitor, to cytochrome P450 enzymes. A, type I difference spectrum; B, type II difference spectrum and C, reverse type I difference spectrum.

---

7 Prof. P. Swart, Biochemistry Department, University of Stellenbosch, South Africa.
The cytochrome P450 content of ovine adrenal mitochondrial and microsomal preparations was determined by the carbon monoxide induced difference spectra [Omura and Sato, 1964]. The P450 enzyme content of the adrenal cortical fractions was determined using the millimolar extinction coefficient as described by Omura and Sato (1964). The carbon monoxide induced difference spectra of solubilised cytochrome P450 in the mitochondrial and microsomal adrenocortical fractions are shown in the Figures 2A and B, respectively. The absorbance at 450 nm is allowed to develop completely since the intensity increases with time after the addition of sodium dithionite. The slow formation of the peak at 450 nm is due to the slow reduction of cytochrome P450. These spectra exhibit peaks at about 450 nm and deep troughs at about 410 nm.

Figure 2. Sodium dithionite reduced carbon monoxide difference spectra of the ovine adrenocortical mitochondrial preparation (A), [cytochrome P450] = 0.78 μM (1.189 nmol P450/mg protein) and the ovine adrenocortical microsomal preparation (B), [cytochrome P450] = 0.442 μM (0.075 nmol P450/mg of protein).

The P450 enzyme content of the adrenocortical mitochondria was 1.189 nmol P450/mg protein and that of the microsomal fraction was 0.075 nmol P450/mg of protein. The mitochondrial preparation contained CYP11A, CYP11B1 and CYP11B2 while the microsomal fraction contained the CYP17, CYP21 and 3βHSD steroidogenic enzymes. Due to unfavourable experimental conditions, such as the use of ionic detergents, cytochrome P450 may be converted to cytochrome P420, its inactive form, displaying a peak at 420 nm. The peak observed in Figure 2B at 424 nm is due to cytochrome b5. Cytochrome b5 can mediate the transfer of the second
electron to the oxygenated cytochrome P450 complex and may be involved in the regulation of adrenal microsomal steroid hydroxylase activities by changing the rates of CYP21 and CYP17 reactions. When cytochrome b5 is reduced by dithionite, the Soret maximum shifts to 423 nm [Yang and Cederbaum, 1994].

4.3.1 Inhibition of cytochrome P450 enzymes by S. frutescens extracts

4.3.1.1 Spectral assays

Aqueous and methanol extracts of S. frutescens were dried and redissolved in water and 50 µl aliquots were subsequently used to carry out all spectral assays. It was found that 5 µl of the extracts (0.41 mg/ml and 0.24 mg/ml final concentration of the plant material in the presence of the methanol and aqueous extract, respectively) did not exhibit any marked inhibition while complete inhibition of the cytochrome P450 enzymes was observed in the presence of 100 µl (8.2 mg/ml and 4.8 mg/ml, respectively) of the extracts. The recommended daily dose for an 80 kg individual is 720 mg (9mg/kg/day) dried plant material [Seier et al., 2002] based on the recommendations of the commercially available S. frutescens tablets. Aliquots (50 µl) of the methanol and aqueous extracts used in the in vitro assays contain 4.1 mg and 2.4 mg of the dried plant material, respectively, which is substantially less than the in vivo recommended dosage.

Spectral Assay A

The microsomal preparation was incubated with 50 µl (methanol and aqueous extracts contain 4.1 mg and 2.4 mg of the dried plant material, respectively) extract to determine if compounds present in the plant extracts interact with the cytochrome P450 enzymes. It would appear that, with the addition of the aqueous extract, the cytochrome P450 enzymes exhibit a reverse type I difference spectrum (Figure 3) with an absorbance maximum at 414 nm. The absorbance minimum, was, however, not clearly detectable as a result of the pigmentation of the extract. The difference spectrum induced by the methanol extract could not be clearly detected due to interference of pigments in this absorbance range.

---

8 Personal communication Prof. P. Swart, Biochemistry Department, University of Stellenbosch, South Africa.
The induction of the reverse type I spectrum firmly suggests that compound(s) in the aqueous extract interact with the microsomal P450 enzymes. It can thus be deduced that compound(s) influence the ferric iron, displacing the water molecule in the active site resulting in an increase in the low spin state of the ferric iron. Furthermore, it is unlikely that the reverse type I substrates bind directly to the heme to displace the water molecule and rather bind to a site in the heme pocket [Lewis 1996].

Figure 3. Reverse type I spectrum of microsomal cytochrome P450 enzymes ([cytochrome P450] = 0.35 μM) induced by the aqueous extract, 50 μl (final concentration 2.4 mg/ml).

Spectral Assay B

Since the aqueous extract interacted with the P450 enzymes, the effect of this interaction on the binding of native steroid substrates to the enzymes was investigated. The binding of deoxycorticosterone (DOC) to CYP11B1, a mitochondrial P450 enzyme, induced a type I spectrum as illustrated in Figure 4A. Although the binding of the methanol extract to the P450 enzymes could not be shown the inhibition of pregnenolone (PREG) and progesterone (PROG) binding to the microsomal P450 enzymes was nevertheless investigated and results are summarized in Figure 4B. DOC binding to the mitochondrial P450 enzymes was inhibited (> 60%) by both extracts (P < 0.005). Inhibition of PREG binding (<10%) was not significant (P = 0.25) while PROG (>52%) binding to the microsomal enzymes was inhibited by both the methanol and aqueous extracts (P < 0.02).
4.3.1.2 Inhibition of steroid conversion

Conversion Assay A

The interactions of the extracts with the P450 enzymes prompted further investigations into the influence of the extracts on the catalysis of steroid substrates by both the ovine adrenal microsomal enzymes, CYP17 and CYP21. Although there was strong inhibition of DOC binding to the mitochondrial P450 enzymes, the influence of the extracts on the catalytic activity of CYP17 and CYP21 was investigated since the inhibition of binding results pointed to different inhibitory activity towards the two microsomal enzymes. Furthermore, CYP17 is at a branch point in glucocorticoid and androgen biosynthesis. Inhibition of the lyase activity of CYP17 would result in the production of the glucocorticoids. Inhibition of CYP21 activity would inhibit the production of the glucocorticoids and shift steroid biosynthesis to the production of androgens.
With the addition of PROG to the microsomal preparation both the P450 enzymes, CYP17 and CYP21, catalyse the conversion of the steroid substrate. CYP17 converted PROG to 17-OH-PROG, while CYP21 converted both PROG and 17-OH-PROG to DOC and deoxycortisol, respectively, as shown in Figure 5A where conversion was monitored in the absence of extracts. Five minutes after the initiation of the reaction, 23 % PROG was converted to DOC, 37 % was converted to deoxycortisol, 10 % PROG was represented by 17-OH-PROG while 30 % PROG remained. HPLC analysis of the steroid metabolites is illustrated in Figure 7A. In the presence of 50 µl methanol and aqueous extracts (final concentration 4.1 mg/ml and 2.4 mg/ml, respectively), the steroid metabolism was inhibited as illustrated in Figure 5B. DOC metabolism appears most markedly inhibited by both methanol (93.6 %) and aqueous (71 %) extracts suggesting that compounds in the extracts inhibit CYP21 more than CYP17 (P < 0.003). The inhibition of DOC formation by the aqueous extract was three times greater than the inhibition of 17-OH-PROG and the inhibition of deoxycortisol (45 %) formation is also greater (P < 0.01) than the inhibition of 17-OH-PROG (21 %). In the presence of the methanol extract, however, 17-OH-PROG formation is inhibited more than deoxycortisol formation, 68 %, and 50 %, respectively. Thus it would appear that the aqueous extract is a more potent inhibitor of CYP21 than the methanol extract, when compared to their inhibitory effects on CYP17.

Figure 5. (A) Metabolism of PROG (10 µM) by ovine adrenal microsomes (cytochrome [P450] = 0.35 µM) after initiation with NADPH (1mM). (B) Percentage inhibition of steroids (after 10 min) in the presence of 50µl methanol and aqueous extracts (final concentration 4.1 mg/ml and 2.4 mg/ml, respectively). Results are presented as the mean, error bars represent SEM and n = 3.
In most species studied to date, CYP17 catalyses the conversion of PREG to 17-OH-PREG and dehydroepiandrosteredione (DHEA). Ovine CYP17, however, does not exhibit significant lyase activity for 17-OH-PREG, as seen when PREG was added to ovine adrenal microsomes, in the presence of trilostane, a 3βHSD inhibitor (Figure 6A). HPLC analysis shows that only 17-OH-PREG is formed and negligible amounts of DHEA was detected (Figure 7B). After 14 min, 32.6% PREG was converted to 17-OH-PREG, this conversion was significantly inhibited \((P < 0.01)\) in the presence of 50 µl methanol and aqueous extracts (final concentration 4.1 mg/ml and 2.4 mg/ml, respectively) by 77% and 49%, respectively (Figure 6B).

![Figure 6](image)

**Figure 6.** (A) Metabolism of PREG (10 µM) by ovine adrenal microsomes (cytochrome [P450] = 0.35 µM) after initiation with NADPH (1mM). (B) Percentage inhibition of steroids in the presence of 50 µl methanol and aqueous extracts (final concentration 4.1 mg/ml and 2.4 mg/ml, respectively). Results are presented as the mean, error bars represent SEM and \(n = 3\).

![Figure 7](image)

**Figure 7.** HPLC analysis of steroid metabolism by ovine adrenal microsomes at 10 min. (A), PROG metabolism (10 µM). Peak 1, PROG (23 min); peak 2, 17-OH-PROG (19.5 min); peak 3, DOC (16.8 min); and peak 4, deoxycortisol (10 min). (B) PREG metabolism (10 µM). Peak 1, PREG (7.5 min); peak 2, 17-OH-PREG (3.5 min); and peak 3, DHEA (2.2 min).
The aqueous extract similarly inhibited the conversion of PREG (49 %) and PROG (49 %). The methanol extract also inhibited PREG (77 %) and PROG (71 %) metabolism similarly. Interestingly, although the extracts did not inhibit PREG binding to the microsomal P450 enzymes, PREG metabolism was inhibited.

**Conversion Assay B**

COS 1 cells were transfected with cDNA encoding CYP17 and CYP21 to investigate the inhibitory effects of *S. frutescens* extracts on the individual cytochrome P450 enzymes. To ensure the inhibition of steroid conversion observed was a result of inhibition of the enzyme and not a decrease in viability of the cells, the protein concentration of the attached cells was determined. If the presence of the extracts resulted in the death of the COS1 cells, the cells would have detached from the surface of the dish resulting in a lower protein concentration compared to that of the control. The protein concentration of the control plate averaged at approximately 0.587 mg/ml while in the presence of 50 µl of the extracts the protein concentration was an average of 0.609 mg/ml. There were no significant differences in protein concentrations observed (P = 0.7) in the presence of 50 µl of the extracts, suggesting that the presence of the extract did not reduce the viability of the cells, thus the presence of the extracts inhibited the enzymes and were not toxic.

The CYP17 enzyme catalyses the conversion of both PROG and PREG to their respective hydroxysteroids in all species. The lyase activity of the CYP17 enzyme, which converts 17-OH-PREG to DHEA and 17-OH-PROG to androstenedione (A4), is however species specific and underlies the complexity of this enzyme in the regulation of steroid biosynthesis. The hydroxylase activity for PREG and PROG has been shown for all species investigated to date but the lyase activity for the hydroxysteroid intermediates differs [Swart 1998]. For example, human and bovine CYP17 catalyses the conversion of 17-OH-PREG to DHEA but not the conversion of 17-OH-PROG to A4 [Chung et al., 1987; Zuber et al., 1986]. Guinea pig CYP17, in contrast, catalyses the conversion of 17-OH-PROG to A4, but not the conversion of 17-OH-PREG to DHEA. Rat, mouse and chicken CYP17 catalyses the lyase reactions of both 17-OH-PREG and 17-OH-PROG [Fevold et al., 1989]. Interestingly, although ovine CYP17 does not exhibit lyase
activity for 17-OH-PREG in microsomal preparations, it does catalyse the reaction in HEK293 cells [Swart et al., 2002].

As a result of these distinct catalytic differences, the influence of *S. frutescens* extracts on the catalytic activity of CYPI7 in different species was investigated; baboon CYPI7, ovine CYPI7 and human CYPI7. Since PROG and 17-OH-PROG are also substrates for the CYP21 enzyme which catalyses the conversion of PROG to DOC and 17-OH-PROG to deoxycortisol, the influence of *S. frutescens* extracts on the activity of bovine CYP21 was also assayed.

Figure 8. Percentage inhibition of PREG (1μM) and metabolites in COS 1 cells expressing baboon CYP17, in the presence of methanol and aqueous extracts (50 μl; final concentration 4.1 mg/ml and 2.4 mg/ml, respectively; and 25 μl; final concentration of 2.05 mg/ml and 1.2 mg/ml, respectively). Results are presented as the mean, error bars represent SEM and n = 3.

Baboon CYP17 converted PREG to 17-OH-PREG and DHEA. In the absence of inhibitor 38 % PREG was converted to 17-OH-PREG and 42 % to DHEA after 24 h. In the presence of 50 μl methanol (4.1 mg/ml) extract, both the lyase and hydroxylase activity of baboon CYP17 are inhibited. However, in the presence of 25 μl methanol extract (2.05 mg/ml) the hydroxylase activity is more significantly (P < 0.001) inhibited (Figure 8). The aqueous extract also inhibited the production of DHEA to a lesser degree than the production of 17-OH-PREG (81.9 % inhibition), suggesting that compounds in the aqueous extract have a greater potential to inhibit the hydroxylase activity (P < 0.01) of CYP17 (Figure 8). With respect to the plant material
extracted, 25 µl of the methanol extract is comparable to the concentrations of plant material in 50 µl of the aqueous extract, 2.05 mg/ml and 2.4 mg/ml, respectively. Thus, from results presented in Figure 8, the aqueous extract exhibits stronger inhibition of CYP17 (P < 0.05) than the methanol extract. The inhibition of the hydroxylase activity in the presence of 25 µl methanol extract and 50 µl aqueous extract is similar while the inhibition of the lyase activity of the enzymes is four times more in the presence (P < 0.02) of the aqueous extract.

![Figure 9. Percentage inhibition of steroids in COSI cells expressing ovine CYP17 in the presence of 50 µl methanol and aqueous extracts (final concentration 4.1 mg/ml and 2.4 mg/ml, respectively). (A) PREG (1 µM) and metabolites, (B) PROG (1 µM) and metabolites. Results are presented as the mean, error bars represent SEM and n = 3.](image)

In the COS1 cells, ovine CYP17 catalysed the conversion of both PROG and PREG to their respective C19 steroids via their respective hydroxysteroid intermediates. Ovine CYP17 converted 44 % PREG to DHEA after 8 h, while the production of A4 from 17-OH-PROG was detectable only after ~12 h. After 24 h, 26.5 % PROG was metabolised to A4 and 17.5 % to 17-OH-PROG, in the absence of the extracts. Figure 9 illustrates the inhibition of the metabolism of PREG and PROG by both the extracts. The methanol extract showed no significant difference in the inhibition of the two substrates (P = 0.126). In addition, although the methanol extract appears to inhibit the lyase and hydroxylase activity of the CYP17 enzyme to a similar degree, assays carried out at lower concentrations of methanol extracts would determine the extracts’ true inhibitory potential of the hydroxylase/lyase activity of the enzyme. Since the aqueous
extract shows significant inhibition \((P < 0.002)\) of the formation of the hydroxysteroid intermediates, it would appear that compound(s) present in the aqueous extract exhibited a greater inhibitory effect on the hydroxylase activity of ovine CYP17.

Figure 10. Percentage inhibition of steroids in COS1 cells expressing human CYP17 in the presence of methanol and aqueous extracts \((5 \mu l, \text{ final concentration } 0.41 \text{ mg/ml and } 0.24 \text{ mg/ml, respectively; and } 50 \mu l, \text{ final concentration } 4.1 \text{ mg/ml and } 2.4 \text{ mg/ml, respectively). (A) PREG (1 \mu M) and metabolites and (B) PROG (1 \mu M) and metabolites. Results are presented as the mean, error bars represent SEM and } n = 3.\)

Figure 11. HPLC analysis of PROG metabolism by human CYP17 at 6 h. Peaks on the chromatogram are: 1, PROG (20 min); 2, 17-OH-PROG (14.3 min); 3, 16-OH-PROG (7.3 min).

Human CYP17 exhibited both 17α-hydroxylase and lyase activity, converting PREG to 17-OH-PREG and subsequently DHEA. After 24 h, 19 % PREG was converted to 17-OH-PREG and 25 % to DHEA. In the presence of \(S. \text{ frutescens}\) extracts, PREG and PROG metabolism were
inhibited as illustrated in Figure 10A and 10B, respectively. The methanol extract inhibited both the hydroxylase and lyase activity of human CYP17 to a similar degree. The aqueous extract also had no significant difference ($P = 0.065$) in inhibitory activity of the two activities of the enzymes (Figure 10A). Progesterone was converted to its hydroxysteroid intermediate and androstenedione was not detected. Human CYP17 exhibits both 17α-hydroxylase activity and 16α-hydroxylase activity with PROG thus being converted to both 17-OH-PROG and 16-OH-PROG [Swart et al., 1993]. HPLC analysis of these steroid metabolites is presented in Figure 11. The conversion of PROG was inhibited in the presence of both the methanol (71 %) and aqueous extracts (57 %), both with a stronger inhibition ($P < 0.05$) of the 16-hydroxylase activity.

![Figure 12. Metabolism of PROG (1 μM) by bovine CYP21 expressed in COSI cells. (A) HPLC analysis of PROG metabolites after 6 h. Peak 1 is PROG (22.7 min) and peak 2 is DOC (19.5 min). (B) Percentage inhibition of PROG metabolites in the presence of 50 μl of the methanol and aqueous extracts (final concentration 4.1 mg/ml and 2.4 mg/ml, respectively). Results are presented as the mean, error bars represent SEM and $n = 3$.](image)

Bovine CYP21 converts both PROG and 17-OH-PROG to DOC and deoxycortisol, respectively. When PROG was given as a substrate, ~50% was converted to DOC after 24 h. HPLC analysis of steroid metabolites assayed in the absence of extracts is shown in Figure 12A. In the presence of methanol and aqueous extracts, PROG conversion was inhibited by 95 % and 66 %, respectively (Figure 12B).
4.3.1.3 Liquid chromatography-Mass spectrometry of *S. frutescens* extracts

LC-MS analyses of the methanol and aqueous extracts showed that the samples comprised a similar but complex mixture. Marked differences in the water extracts, prepared at 100°C and 50°C, were noticed. It is evident that the aqueous extraction carried out at 100°C, yielded a similar LC-MS elution profile as the methanol extract. Water extraction under reduced pressure at 50°C, however, did not yield the two prominent fractions seen at retention times between 5.38 and 6.06 min for the methanol and the aqueous extracts prepared at 100°C.

![UV Chromatogram](image)

**Figure 13.** Analysis of methanol extracts of *S. frutescens* (A) UV chromatogram and (B) Total ion chromatogram (ESI negative) 0-15 min.
Figure 14. Analysis of aqueous extracts of *S. frutescens* (A) UV chromatogram and (B) Total ion chromatogram (ESI negative) 0-15 min.

Figure 15. Analysis of aqueous extracts of *S. frutescens* prepared at 50°C under reduced pressure (A) UV chromatogram and (B) Total ion chromatogram (ESI negative) 0-15 min.
Interestingly the two components, identified by Tai et al. (2004), with molecular weights of 740 (peaks at 5.38 min and 5.58 min) and 724 (peaks at 6.06 min), were confirmed by negative ion electrospray mass spectrometry (Figure 13 and 14). These two compounds were not observed in the aqueous extract carried out at the lower temperature (Figure 15). The combination of electrospray mass spectrometry in the negative mode, combined with UV may indicate the presence of flavonoids. Phenolic compounds, such as flavonoids, have the ability to be negatively ionized while at the same time some conjugated double bonds or aromatic rings present in the structure absorb in the aromatic region. The hydrophobic compounds eluting later exhibiting little absorbance in the UV range would include the more complex flavonoids and possibly the triterpenoids.

4.3.2 Inhibition of cytochrome P450 enzymes by bioactive compounds in S. frutescens

In S. frutescens, L-canavanine, pinitol, GABA, triterpenoids as well as flavonoids have been identified as bioactive compounds having medicinal properties. An investigation was therefore carried out to determine whether these compounds were able to interact with cytochrome P450. The ability of these compounds to induce a difference spectra as well as their ability to influence substrate binding was assayed. The influence of the triterpenoid fraction on the catalytic activity of cytochrome P450 was assayed in adrenal microsomes.

4.3.2.1 Spectral assays

Spectral Assay A

Only the triterpenoid fraction purified from S. frutescens was able to induce a significant Type II difference spectra at the concentrations tested (Figure 16). The Type II induced difference spectrum suggests that the triterpenoids bind directly to heme iron.
Figure 16. Type II difference spectrum of microsomal cytochrome P450 enzymes ([cytochrome P450] = 0.6 μM) induced by the triterpenoid fraction (0.25 mg).

Spectral Assay B

The bioactive compounds were tested for activity using 0.15 mg compound/0.6 μM cytochrome P450. Substrate binding was inhibited by the triterpenoid fraction only, as was shown by the reduction of the amplitude of the PROG-induced type I difference spectrum in adrenocortical microsomes. Figure 17 summarizes the inhibition of PROG binding to the microsomal P450 enzymes in the presence of the bioactive compounds. PREG binding was not inhibited by any of the compounds and the result is therefore not included. However, at a higher concentration, 1.5 mg, the triterpenoid fraction was able to reduce the PREG-induced difference spectrum illustrated in Figure 18B. Interestingly, the flavonoids, which are also similar in structure to the adrenal steroid hormones, did not inhibit binding when 0.15 mg was assayed. Higher concentrations could, however, not be tested due to a limited supply of the flavonoid fraction.

Figure 17. Percentage inhibition of PROG (3.2 μM) binding to microsomal cytochrome P450 enzymes (cytochrome [P450] = 0.35 μM) by 0.15 mg bioactive test compound. Results are presented as the mean, error bars represent SEM and n = 3.
Figure 18. Inhibition of substrate-induced type 1 difference spectra in ovine adrenal microsomes (cytochrome [P450] = 0.35 μM) by 1.5mg triterpenoid fraction. Inhibition of (A) (---), PROG (3.2 μM), (-----) PROG + triterpenoid and (B) (--), PREG (3.2 μM), (-----) PREG + triterpenoid.

Figure 19. Percentage inhibition of PROG (3.2 μM) and PREG (3.2 μM) binding to ovine adrenocortical microsomes (cytochrome [P450] = 0.35 μM) in the presence of varying concentrations of triterpenoids. Results are presented as the mean, error bars represent SEM and n = 3.

Figure 18 illustrates the reduction in amplitude of (A) PROG- and (B) PREG-induced difference spectra by the triterpenoid fraction. Figure 19 illustrates that the triterpenoids exhibit a greater inhibitory effect on the binding of PROG (P < 0.05) to cytochrome P450 — a 50 % inhibition of PROG binding is obtained in the presence of 0.6 mg triterpenoids while a 40 % inhibition of PREG binding necessitates 1.5 mg triterpenoids.
4.3.2.2. Inhibition of steroid conversion

Conversion Assay A

Since the triterpenoid fraction showed significant inhibition of steroid binding to the cytochrome P450 enzymes, the conversion of steroids in ovine adrenal microsomes was assayed in the presence of this fraction.

![Conversion Assay A](image)

Figure 20. Percentage inhibition of PROG (10 μM) and PREG (10 μM) conversion in ovine adrenal microsomes (cytochrome P450 = 0.35 μM) by 0.3 mg triterpenoid. Results are presented as the mean, error bars represent SEM and n = 2.

Conversion was monitored over a 25 min period and inhibition was observed in the presence of 0.3 mg triterpenoids. PROG conversion was inhibited by 62% and PREG conversion was inhibited by 41% (Figure 20). The metabolites of the conversion assay are not presented, as they were not sufficiently separated to determine their relative concentrations. Due to a limited supply of the triterpenoid fraction the experiment could not be repeated to obtain the required separation.

4.3.3 Inhibition of cytochrome P450 enzymes by *Sutherlandia* tablets

Spectral Assay B

From the previous results it is clear that dried *S. frutescens* plant material contains compounds capable of inhibiting the cytochrome P450 enzymes. Since the manufacturing process of
Sutherlandia in the production of commercially available tablets may influence the activity of the bioactive compounds, the effect of tablet extracts on steroid binding was investigated and results are presented in Figure 21.

![Percentage inhibition of steroid binding](image)

Figure 21. Percentage inhibition of DOC (3.2μM) binding to CYP11B1 (cytochrome [P450] = 0.78 μM), and PROG (3.2 μM) and PREG (3.2 μM) binding to microsomal P450 enzymes (cytochrome [P450] = 0.35 μM), by 50 μl tablet extracts (0.3 mg/ml). Results are presented as the mean, error bars represent SEM and n = 3.

Only the tablets purchased Health Foods were capable of significantly inhibiting only DOC binding (P < 0.02). PREG and PROG binding to the adrenal microsomes were, however, not significantly inhibited by the concentrations of the tablet extracts tested. Low levels of inhibition were observed as only 1 % of extracted compounds from a single tablet were added to the reaction mixture — 3 mg dried plant material was assayed. Since the inhibition of binding of steroid substrates to cytochrome P450 enzymes by these products is very low no deductions with regards to the influence of the manufacturing processes on the bioactivity of the Sutherlandia tablets can be made.

### 4.4 Discussion

Extracts of *S. frutescens* and natural compounds previously identified in the plant interact with CYP11B1, CYP17 and CYP21 as was observed in the assays carried out with the steroidogenic cytochrome P450 enzymes. Test components influenced enzyme activities by binding to the cytochrome P450 enzymes, influencing the binding of endogenous substrates and had an effect on substrate metabolism.
Aqueous extracts, when assayed in adrenal microsomes, induced a reverse type I difference spectrum. The binding of reverse type I inhibitors favours a spin equilibrium shift of the ferric iron to its low-spin form. The sixth axial ligand may therefore be more firmly bound rather than displaced, as with type I substrates. The exact nature of the interaction is unknown. Since most reverse type I inhibitors contain oxygen and/or nitrogen, there may be hydrogen bonded interactions between the inhibitor and the water sixth ligand. If, in the unlikely event, the reverse type I inhibitor binds to the heme, the interaction is too weak to induce a shift in the absorption maximum [Lewis, 1996]. It is most probable that these inhibitors bind in the hydrophobic pocket of the P450 enzyme to a second site different from that occupied by type I substrates.

While only the aqueous extract was able to induce a detectable reverse type I spectrum, both aqueous and methanol extracts were able to inhibit the binding of DOC and PROG to the P450 enzymes in the mitochondria and microsomes, respectively. The inhibition of PREG binding to the microsomal P450 enzymes was, however not significant ($P = 0.25$). Although the binding of PREG to CYP17 was not inhibited, \textit{in vitro} investigations revealed that both \textit{S. frutescens} extracts were able to inhibit the metabolism of both PROG and PREG by the microsomal P450 enzymes. This suggests that bioactive compound(s) present in the extracts may bind to a site in the active pocket other than that occupied by PREG and thus not inhibit PREG binding. Since PROG and DOC binding was inhibited, bioactive compounds in \textit{S. frutescens} may bind to the same site or in the same orientation as the natural substrates, or binding may bring about structural changes of the enzyme which subsequently hampers the binding of the natural substrates.

Both the hydroxylase and lyase activities of the P450 enzymes are influenced by \textit{S. frutescens} as was shown with the inhibition of PROG ($\sim 50\%$) and PREG ($\sim 70\%$) metabolism \textit{in vitro}, by the aqueous and methanol extracts. When PROG metabolism was assayed the inhibition of CYP21 was greater than the inhibition of CYP17 by both the aqueous and methanol extracts since the inhibition of DOC was significantly higher ($P < 0.003$) than the inhibition of 17-OH-PROG by the extracts.

Since the microsomes contain both CYP21 and CYP17 enzymes, the biological activity of \textit{S. frutescens} was investigated in COS 1 cells. The possibility that the extracts could influence the viability of the cells was considered. Numerous cytotoxic and cell viability assays are currently
available. The choice of the appropriate assay will depend on the cell type, the information required and the cost involved [Celis, 1998]. Since the assay described in this study set out to determine whether there was a difference between steroid metabolism in transfected COS 1 cells in the absence and in the presence of *S. frutescens* extracts, the measurement of the number of viable cells or the number of dead cells may be irrelevant. If however, the mechanism of cell death i.e. apoptosis and/or necrosis was being investigated, the duration of exposure to *S. frutescens* extracts and the concentration of compounds in the extracts, become critical [Riss and Moravec, 2004]. For this study however the determination of the viability of the COS 1 cells (cells remaining attached for the duration of the assay) was assayed as described using a relative inexpensive and readily available protein determination. 

CYP17 from different species was investigated and the influence of *S. frutescens* extracts on the catalytic activity of this enzyme differed across species. When the substrate inhibition by the aqueous and methanol extracts was greater than 80% the inhibition on the lyase and hydroxylase activity was similar. Different concentrations of dried *S. frutescens* plant material are present in the methanol and aqueous extract, 82 mg/ml and 48 mg/ml, respectively. When comparing methanol and aqueous extract with similar concentrations of extracted plant material, the aqueous extract exhibit a greater inhibition of PREG metabolism by baboon CYP17 than the methanol extract (P < 0.05). Both the aqueous and methanol extract exhibited a greater inhibition on the hydroxylase activity of CYP17 than the lyase activity (P < 0.01). In addition, the inhibition of the hydroxylase activity of ovine CYP17 by the aqueous extract was greater than the inhibition of the lyase activity of the enzyme (P < 0.002). Subtle differences in the inhibition of the 17- and 16-hydroxylase activity of human CYP17 by the methanol and aqueous extract were observed. Both extracts exhibited a greater inhibition (P < 0.05) on the 16-hydroxylase activity of human CYP17. The hydroxylase and lyase activities were influenced in different ways by the extracts possibly suggesting different mechanisms of action of inhibition of the enzymes. Furthermore, there were differences in inhibition of the PROG and PREG substrates that suggest there may be many bioactive compounds present in *S. frutescens* capable of inhibiting the catalytic activity of the P450 enzymes. Analysing HPLC fractions of *S. frutescens* extracts for inhibitory activity would identify the presence of bioactive compounds in *S. frutescens* that interact with the P450 enzymes.

9 http://www.jingmei.com/site/zt/Roche/FuGENE%206%20Feature%20Presentation.pdf
Compounds may influence the binding of the substrate in the active site and possibly also the binding of other role players involved in the catalytic activity of the enzyme. Compounds may bind to residues on the surface of the P450 enzyme, which may be important for the binding of the redox partners. This interaction would still allow the endogenous steroid to bind to the enzyme but may inhibit substrate conversion by interfering with the interaction of the P450 enzyme with its redox partners and blocking the electron transport chain. In addition, compounds may inhibit the conversion by interacting with molecular oxygen or the phospholipid environment, which both play a role in catalytic activity of the P450 enzymes.

The triterpenoid and flavonoid compounds present in S. frutescens, have similar structures to the steroidogenic enzymes and may thus possess corticomimetic activity. However, only the triterpenoid fraction was able to bind in the active pocket of the P450 enzyme, inducing a type II difference spectrum. Type II inhibitors bind directly to the heme iron, replacing the water molecule as the sixth axial ligand. The inhibitor-induced type II difference spectrum was, however, not observed in the presence of the S. frutescens extracts. It can be hypothesized that the triterpenoids are present at low concentrations and therefore unable to induce a detectable type II spectrum. Since the triterpenoids are able to induce a type II difference spectrum and the aqueous extract induced a reverse type I spectrum, the presence of more than one bioactive compound in S. frutescens with respect to inhibition of the P450 enzymes, is confirmed.

The triterpenoids were able to inhibit the binding of PROG and PREG to the microsomal P450 enzymes as a result of ligating directly to the heme. L-canavanine, GABA, pinitol and the flavonoids were unable to inhibit the binding of steroids to the P450 enzymes at the concentrations tested. It is possible that the flavonoids may not be strong inhibitors of P450 enzymes but the lack of inhibition by the flavonoids may be a result of the low concentrations used in the assays. Due to a limited supply of the flavonoids, higher concentrations could not be tested. LC-MS analysis identified the presence of aromatic phenolic compounds, with molecular masses of 740 and 724, in both the methanol and aqueous extract. From the peak intensities it appears that the aqueous extraction was more successful at extracting these compounds than methanol extraction. However further HPLC analysis is required to confirm the relative quantities. An aqueous extract was prepared at 50°C under reduced pressure and the LC-MS
analysis, interestingly revealed that this extract did not contain the compounds with molecular masses of 740 and 724. This extract was assayed in ovine adrenal microsomes and exhibited negligible inhibition on the catalytic activity of the P450 enzymes (results not shown). This finding suggests that the phenolic compounds with molecular masses of 740 and 724, contribute to the inhibitory activity observed in the presence of the methanol and aqueous extracts. In some instances, the aqueous extract prepared at a lower temperature, appeared to stimulate the catalytic activity of the P450 enzymes present in the microsomal preparation (results not shown). Natural plant products have been identified to stimulate the activity of liver P450 enzymes. Thus, it is possible that compounds present in *S. frutescens* are capable of stimulating the catalytic activity of the P450 enzymes. Analysis of HPLC fractions of the extracts may be able to identify the compounds with stimulatory effects.

The inhibition of the metabolism of PROG and PREG by the microsomal P450 enzymes in the presence of the triterpenoid fraction may be attributed to the fact that they bind directly to the heme iron. High concentrations of the triterpenoid fraction ~ 0.4 mM was however required to achieve an inhibitory effect. PROG binding and conversion was inhibited to a greater degree (P < 0.05) than PREG binding and conversion. It has been suggested that the triterpenoids are present in relatively low concentrations in *S. frutescens* plant material [Van Wyk, 2004]. HPLC analysis of the extracts and triterpenoid fractions would allow the quantification of the concentrations of triterpenoids present in the plant material and extracts to allow a direct comparison of the results obtained by the extracts and triterpenoids. One can, however, estimate the concentrations of the flavonoid and triterpenoid fractions which were assayed using an average molecular mass for flavonoids and triterpenoids of 730 and 690, respectively. It can therefore be concluded that triterpenoid concentrations of ~0.4 mM and ~2 mM were used to achieve a 50% inhibition of PROG and PREG binding, respectively. These concentrations are exceedingly high compared to the concentration present in the plant material. This would underlie the hypothesis that it is not only a single compound interacting with the P450 enzymes, but rather many compounds acting synergistically.

The commercially available tablets assayed, contain 300 mg dried plant material and the concentration of dried plant material used in spectral assay B was much similar to the concentrations of dried plant material in the extracts, 3 mg/ml versus 4.1 mg/ml or 2.4 mg/ml.
The tablet extracts were prepared at a temperature of 37°C and may therefore contain fewer of the bioactive compounds. As a result, inhibition of steroid binding to the mitochondrial and microsomal P450 enzymes was much lower than that observed in the presence of the extracts. In addition, tablets purchased from different manufacturers showed negligible differences in inhibition. The active compounds in plants are usually secondary metabolites and their concentrations vary as a result of season, time of day, soil type and other environmental factors. The bioactivity of these products may be influenced by the harvesting of different plant specimens, post-harvesting storage of plant material and by the manufacturing procedures employed for the development of the tablets. Higher concentrations of tablet extracts would have to be assayed to allow conclusions to be made about the influence of manufacturing procedures on the activity of the compounds.

In summary, *S. frutescens* contains bioactive compounds capable of inhibiting the activity of the CYP11B1, CYP21 and CYP17 enzymes involved in the biosynthesis of steroids at concentrations lower than the recommended dosage. A traditional decoction as well as commercially available tablets of *S. frutescens*, posses the inhibitory activity and are thus effective dosage forms for the inhibition of the steroidogenic P450 enzymes and the subsequent reduction of cortisol biosynthesis. These results provide insight into the mechanism with which *S. frutescens* is able to reduce glucocorticoid levels and provides preliminary scientific appraisal for the stress relieving properties of the plant.
The Effect of Sutherlandia frutescens on Steroidogenesis:
Confirming Indigenous Wisdom

D. Prevoo, C. Smith, P. Swart, and A. C. Swart

Department of Biochemistry and Department of Physiological Sciences, University of Stellenbosch, Stellenbosch, South Africa

ABSTRACT

Sutherlandia frutescens (Cancer bush), a Southern African indigenous plant, is traditionally used to treat stress related maladies linked to the endocrine system. Extracts of the shrub were used to investigate the claimed stress-relieving properties of the shrub. Dysregulation of the stress response is associated with elevated glucocorticoid levels. A model of chronic intermittent immobilization stress was investigated in 40 adult male Wistar rats to determine the effect of Sutherlandia. Immobilization stress resulted in increased corticosterone levels in the control group while rats receiving Sutherlandia extract showed significantly decreased corticosterone levels ($P < 0.005$). Since the biosynthesis of glucocorticoids in the adrenals is catalyzed by the cytochrome P450-dependent enzymes, the influence of Sutherlandia extracts on adrenal steroidogenesis was determined in ovine adrenocortical microsomes and mitochondria, using spectral binding and enzyme conversion assays. Water extracts showed inhibition of substrate binding to cytochrome P450 21-hydroxylase (CYP21) by 38% and cytochrome P450 11β-hydroxylase (CYP11B1) by 60%. The conversion of progesterone and pregnenolone was inhibited by 34% and 30%, respectively. Subsequent extractions with chloroform and methanol showed inhibition of substrate binding and conversion with hydrophobic compounds exhibiting a greater inhibitory effect on deoxycorticosterone binding to CYP11B1 (30%) and on progesterone binding to CYP21 (50%). The inhibition of binding of pregnenolone
to CYP17 by the chloroform extract was 62%, with negligible inhibition by the methanol extract. The chloroform extract showed a greater inhibitory effect than the methanol extract on progesterone and pregnenolone metabolism (20%−50%).

Key Words: Sutherlandia frutescens; Steroidogenesis; Cytochrome P450.

INTRODUCTION

Many indigenous plants have long been used as traditional remedies for a variety of ailments. *Sutherlandia frutescens* (Cancer bush) is one such plant and is used to treat many maladies, including stress related illnesses (1,2). There is, however, little scientific evidence to support these claims of therapeutic effects. To date few active compounds have been isolated and characterized from *Sutherlandia*. Some of these compounds have been shown to have anticancer, antiinflammatory, and antiviral properties and are capable of inhibiting nitric oxide synthase (3–6). *Sutherlandia* has been anecdotally reported to bring relief to HIV/AIDS and cancer patients, who are subject to a chronic increase in blood cortisol levels (7,8). Since side effects are not well established, a toxicity study in vervet monkeys was undertaken. *Sutherlandia* was shown to have no toxic or side effects with regards to the parameters measured (9).

Excessive and sustained cortisol secretion resulting from stress and stress related illnesses has been associated with immunosuppression, cardiovascular disease, and a host of other ailments (7,10). Since adrenal cytochromes P450 are responsible for the biosynthesis of glucocorticoids, cortisol and corticosterone, a possible target tissue for testing the antistress function of *Sutherlandia*, is the adrenal gland. The therapeutic properties of *Sutherlandia* were therefore investigated in Wistar rats and the influence on adrenal steroidogenesis was determined.

The effect of *Sutherlandia* on plasma corticosterone and testosterone levels in adult male Wistar rats, subjected to immobilization stress, was determined by administering extracts intraperitoneally. The influence of *Sutherlandia* on the cytochrome P450 enzymes, which catalyze the biosynthesis of cortisol precursors in the adrenal, was subsequently investigated—the inhibition of substrate binding to these enzymes and steroid metabolism in ovine adrenal microsomes, in the presence of *Sutherlandia*, was determined.

MATERIALS AND METHODS

In Vivo Studies

The effect of *Sutherlandia* was determined in adult male Wistar rats subjected to stress as previously described (1). Forty rats were divided into 4 mass-matched groups (*n* = 10). Two groups were immobilized for 2 h once a day for 28 days in addition to receiving either *Sutherlandia* extract (IS) or placebo (isotonic saline) (IP) and two control groups received either *Sutherlandia* extract (CS) or placebo (CP). An aqueous extract was administered intraperitoneally, 2 mg/0.5 ml twice daily for 28 days. Serum corticosterone and testosterone concentrations were determined by radio
The Effect of *Sutherlandia frutescens* on Steroidogenesis

immunoassays (Biotrak RPA 548, Amersham) and immunoassays (Advia Centaur, Bayer Diagnostics), respectively.

**Preparation of *Sutherlandia* Extracts**

Aqueous extract was prepared by boiling 2.16 g dried *Sutherlandia frutescens* in 275 ml deionized water for 30 min. The extract was lyophilized, redissolved in 13 ml deionized water and subsequently centrifuged at 6000 g for 5 min.

Dried plant material, 18.6 g, was extracted with 250 ml chloroform and methanol for 5 and 8 h, dried and redissolved in 35 ml polyethylene glycol (PEG) 400 (BDH chemicals Ltd, UK) and deionized water, respectively (11).

**Cytochrome P450 Binding and Substrate Conversion Assays**

Ovine adrenal microsomes and mitochondria were prepared as previously described (12). The cytochrome P450 content was determined by the carbon monoxide method using an extinction coefficient ($\varepsilon$) of 91 cm$^{-1}$·mM$^{-1}$ (13).

Spectral assays were carried out and the induced difference spectra recorded between 360 nm and 500 nm (11). Steroid substrates (Sigma Chemical Co. St. Louis, MO, USA) dissolved in ethanol (1.6 mM), were added to the microsomes (0.33 µM P450) and mitochondria (0.78 µM P450) to a final concentration of 3.2 µM. Inhibition of pregnenolone (Preg), progesterone (Prog), and deoxycorticosterone (DOC) binding was measured by a decrease in the induced difference spectrum upon the addition of 50 µl *Sutherlandia* extracts (9).

Adrenal microsomes (0.35 µM P450) were preincubated at 37°C and Prog, 10 µM, and Preg, 10 µM, metabolism assayed in the presence of 50 µl extract, tritiated steroid (PerkinElmer Life Sciences Boston, MA), 0.4 U isocitrate and 1 mM NADPH (Roche, Germany) in a final reaction volume of 1 ml (14). Aliquots, 50 µl, were extracted with dichloromethane and Prog and Preg metabolites were separated on a Novapak® C18 and C8 column, respectively, at a flow rate of 1 ml/min with a Waters (Milford, MA) HPLC coupled to a Waters 700 satellite WISP™ automatic injector and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL) (15).

**RESULTS**

**In Vivo Studies**

Plasma analysis showed that immobilization stress produced significantly increased corticosterone levels ($P < 0.05$) and decreased testosterone levels ($P < 0.05$). These findings are reflected in the significantly higher corticosterone:testosterone ratio seen in the stressed versus the control groups receiving the placebo ($P < 0.05$). In the two groups subjected to stress, the group receiving the *Sutherlandia* supplement showed significantly decreased corticosterone plasma levels ($P < 0.005$). Interestingly, corticosterone levels were significantly higher in the control group receiving the *Sutherlandia* supplement than in the control group receiving the placebo ($P < 0.05$). Both these
Prevo et al.

**Table 1.** Inhibition by methanol and chloroform extracts of steroid binding (0.78 μM P450) and substrate conversion (0.3 μM P450) in ovine adrenal mitochondria and microsomes.

<table>
<thead>
<tr>
<th>% Inhibition of steroid (3.2 μM) binding</th>
<th>% Inhibition of steroid (10 μM) conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DOC</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>59</td>
</tr>
<tr>
<td>Chloroform extract</td>
<td>80</td>
</tr>
</tbody>
</table>

*Not significant.

control groups, CP and CS, had significantly higher testosterone levels than the stressed group receiving the *Sutherlandia* supplement ($P < 0.01$ and $P < 0.05$).

**Cytochrome P450 Binding and Conversion Assays**

The binding of DOC to CYP 11B1 exhibits a type 1 induced spectrum (Fig. 1A). Varying degrees of inhibition on substrate binding was observed in the presence of *Sutherlandia* extracts, indicated by the reduced amplitude in the difference spectra. The aqueous extract inhibited the binding of DOC by 60%. While the metabolism of both Prog and Preg was inhibited, the inhibition of Preg binding was negligible (Fig. 1B). Typical HPLC analyses of steroid metabolites present in the medium after 10 min are shown in Fig. 2.

![Figure 1](http://scholar.sun.ac.za)
The Effect of Sutherlandia frutescens on Steroidogenesis

Steroid binding and conversion was inhibited in the presence of both methanol and chloroform extracts (Table 1). Chloroform extracts displayed greater inhibition of steroid binding and substrate conversion than the methanol extracts, while the latter did not inhibit Preg binding as was previously seen with the aqueous extracts.

**DISCUSSION**

The effects of Sutherlandia treatment on chronic intermittent immobilization stress manifested as a significant decrease the corticosterone response, supporting the indigenous knowledge of Sutherlandia's stress relieving properties. Inadvertently this result may also support the claims that Sutherlandia can improve the quality of life of cancer and HIV/AIDS patients.

The inhibitory effects of Sutherlandia extracts on the cytochromes P450, which catalyze the biosynthesis of cortisol and its precursors, substantiated the above findings. The aqueous extract inhibited substrate binding to the microsomal (CYP17) and mitochondrial (CYP11B1) cytochrome P450 enzymes. Since compounds in the plant material inhibit enzyme/substrate binding, steroid production would ultimately be
affected. However, both CYP17 and CYP21 were inhibited by chloroform and methanol extracts with the hydrophobic compounds showing a far greater inhibitory effect on the biosynthesis of steroid intermediates than the hydrophilic compounds present in both the methanol and aqueous extracts.

Interestingly, basal corticosterone concentrations were significantly elevated in control rats receiving *Sutherlandia* supplements when compared to placebo rats in the absence of an additional stressor. *Sutherlandia* may act as an adaptogen effecting a more functional basal serum corticosterone level. Aqueous extracts prepared under milder conditions, was found to stimulate the conversion of Preg by CYP17 in COS1 cells and in adrenal microsomes (unpublished results).

Our data shows that hydrophilic compounds in the plant extract did not inhibit substrate binding to CYP17 but did, however, inhibit the metabolism of Preg indicating that within the microsomal environment, compounds in *Sutherlandia* may possibly affect other role players in the catalytic reaction. It is clear that *Sutherlandia* contains compounds that affect adrenal steroidogenesis, but these active compounds are possibly complexed in the plant material requiring specific extraction conditions for the dissociation and subsequent activation of compounds.

**ACKNOWLEDGMENTS**

The authors would like to thank the University of Stellenbosch and the NRF for funding, and Prof. Van Wyk for providing the *Sutherlandia* plant material.

**REFERENCES**

Chapter Six

Conclusion

*S. frutescens* is one of 3000 medicinal plants in South Africa and is considered the most profound adaptogenic tonic. Claims that *S. frutescens* can be used in the treatment of the symptoms associated with HIV/AIDS and cancer have prompted scientific investigations into the validation of the anecdotal therapeutic claims of the plant. In addition, *S. frutescens* is reported to improve the quality of life in general and has been used traditionally to treat a multitude of maladies including colds, flu, stomach problems, inflammation and stress [Van Wyk and Wink, 2004].

Psychological and physical stress activates the HPA-axis stimulating the increased production of the glucocorticoids, cortisol and corticosterone. The HPA-axis is intricately regulated by neuroendocrine signals with the glucocorticoids regulating the activity of the HPA-axis by negative feedback. In chronic stress situations, which include psychological stress, HIV/AIDS and cancer, the negative feedback regulation is interrupted resulting in permanently elevated plasma glucocorticoid levels. Prolonged exposure to cortisol has been associated with many adverse effects [Chrousos and Gold, 1998]. The deleterious effects of elevated levels of cortisol may be alleviated by inhibiting the enzymes responsible for the biosynthesis of cortisol. *S. frutescens* may relieve the symptoms of stress and improve the quality of life of cancer and HIV/AIDS patients by inhibiting the catalytic activity of steroidogenic cytochrome P450 enzymes, therefore decreasing circulating glucocorticoid concentrations.

Plasma cholesterol is the steroid substrate for the biosynthesis of cortisol. The adrenal cytochrome P450 enzymes are heme proteins which catalyse the complex reactions involved in steroid hormone biosynthesis. The reactions catalyzed by the enzymes require the transfer of two electrons from NAD(P)H either via cytochrome P450 reductase in the endoplasmic reticulum, or via ferredoxin reductase in the mitochondria. The mitochondrial P450 enzymes, CYP11A, CYP11B1 and CYP11B2, employ an iron sulphur protein, adrenodoxin, and adrenodoxin reductase to transport electrons. The adrenodoxin protein shuttles the electrons from the
reductase to the P450 enzyme. The microsomal P450 enzymes, CYP17 and CYP21, acquire their electrons directly from the reduced reductase. Due to the complex nature of these enzymes, there are many ways to inhibit the catalytic activity of the enzyme. Inhibitory compounds can bind to the active site and induce a conformational change, which prevents binding of the natural steroid substrates. Compounds may also bind to other sites on the enzyme and may thus inhibit the binding of electron transport proteins. The phospholipid environment and the availability of molecular oxygen are other factors that play a role in the catalytic activity of the P450 enzymes, thus inhibitory compounds may interfere with these parameters.

UV/Visible spectrometry revealed that components of *S. frutescens* were able to bind to the P450 enzymes and inhibit the type I difference spectrum induced by the binding of natural steroid substrates. The uncommon reverse type I spectrum was induced by the aqueous extract of *S. frutescens*, suggesting that components of *S. frutescens* bind in the heme pocket of the enzyme to a site different from that occupied by the natural steroid substrates. Compounds that access and bind to the active site, are usually similar in structure to the natural steroid substrates of the enzyme. The flavonoids and triterpenoids present in many plants have a similar structure to these natural steroids. The triterpenoids present in *S. frutescens* were able to elicit a type II spectrum, showing thus that these compounds bind directly to the heme in the active pocket of the enzyme, displacing the sixth axial ligand of the heme. The flavonoid compounds present in *S. frutescens* were unable to induce a difference spectrum or inhibit the binding of natural steroids to the P450 enzymes. It is possible that the flavonoids may not be strong inhibitors of P450 enzymes, but the lack of inhibition by the flavonoids may be a result of the low concentrations used in the assay. The induction of a type II and reverse type I difference spectrum establishes the presence of more than one compound in *S. frutescens* with the potential to interact with the P450 enzymes.

*In vitro* and *in vivo* assays demonstrated that *S. frutescens* extracts were able to inhibit the catalytic activity of the P450 enzymes present in the endoplasmic reticulum of the adrenal cortex. Although the inhibition of PREG binding to the microsomal enzymes by the methanol and aqueous extracts was negligible, both extracts were capable of inhibiting the metabolism of PREG suggesting that components of the extracts possibly interfere with other role players involved in cytochrome P450 catalysis such as the phospholipid environment or the redox partners employed by the enzymes. Both the methanol and aqueous extract inhibited both CYP21
and CYP17 in COS 1 cells. The inhibition of CYP21 was greater than inhibition of CYP17. Furthermore, the degree of inhibition of CYP17 differed across species but both extracts inhibited the hydroxylase activity of CYP17 more potently than the lyase activity of the enzyme.

UV analysis of the methanol and aqueous extracts identified two prominent peaks which absorbed at the wavelength of aromatic compounds and may thus be phenolic compounds present in *S. frutescens* such as the flavonoids. These peaks have molecular weights of 740 and 724, which were determined by negative ion electrospray mass spectral analysis. An aqueous extract of *S. frutescens* prepared at a lower temperature under reduced pressure did not contain these prominent peaks. In addition, the inhibition of the catalytic activity of the microsomal P450 enzymes in the presence of this aqueous extract was negligible. This result suggests that the compounds with molecular weights of 740 and 724 may contribute considerably to the inhibitory effect of *S. frutescens* on the steroidogenic P450 enzymes. Furthermore, the aqueous extract prepared at a lower temperature often stimulated the catalytic activity of the P450 enzymes. Future investigations would involve collecting HPLC fractions of the extracts to determine which compounds are responsible for influencing the catalytic properties of P450 enzymes.

In an investigation in rats, it was shown that rats treated with *S. frutescens* not only exhibited decreased plasma glucocorticoid levels in response to chronic immobilization stress but also increased basal corticosterone levels in non-stressed rats. *S. frutescens* may therefore exert its stress relieving properties by influencing the HPA-axis acting as an adaptogen (a substance which increases the body’s ability to adapt and increase resistance to stress, normalising body functions through allostasis [Smith and Myburgh, 2004]) as well as by down-regulating glucocorticoid production. The inhibition of elevated glucocorticoid levels in response to chronic stress may be attributed to the inhibitory effect of *S. frutescens* on the steroidogenic P450 enzymes. Future investigations would involve examining the anti-stress properties on other potential target tissues such as the hippocampus, hypothalamus and pituitary.

In summary, these results provide preliminary scientific appraisal of the stress relieving properties of the plant by providing insight into the mechanism with which *S. frutescens* is able to reduce glucocorticoid levels. Commercially available tablets also contain the inhibitory activity and are thus an effective dosage form for the inhibition of the P450 enzymes. Although
safety and toxicity reports have suggested that *S. frutescens* plant material is not toxic at nine times the recommended dose, other factors need to be considered before the plant is prescribed for medicinal purposes. It has been reported that *S. frutescens* is able to inhibit the drug metabolizing cytochrome P450 enzymes and thus may influence, delay, compromise or replace the efficacy of conventional medicines when these are used in conjunction with *S. frutescens*. This characteristic of *S. frutescens* highlights the importance of scientific investigations on the medicinal properties of traditional medicine in general. To bring traditional medicine to the level of efficacy and safety where it can be regarded as an acceptable alternative to western healthcare systems requires scientific validation, strict regulation and trained healthcare practitioners.
References


