An investigation into the bioactivity of *Sutherlandia frutescens* (Cancer bush)

Egbichi Ifeanyi M

Thesis presented in partial fulfillment of the requirements for the degree of Masters of Science (Biochemistry) at the University of Stellenbosch

Supervisor: Dr A C Swart
Co-Supervisor: Prof P Swart
Department of Biochemistry,
University of Stellenbosch,
South Africa.
March 2009
Declaration

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

Signature………………………………

Date: 3 March 2009
Summary

This study describes the:

1. preparation of aqueous, methanol and chloroform *S. frutescens* extracts.
2. preparation of ovine adrenal microsomal and mitochondrial P450 enzymes.
3. investigation of the inhibitory effect of the *Sutherlandia frutescens* extracts on steroid substrate binding to the P450 enzymes.
4. effect of *Sutherlandia frutescens* extracts on progesterone, deoxycortisol and deoxycorticosterone metabolism in ovine adrenal microsomes and mitochondria.

Opsomming

Hierdie studie beskryf die:

1. ekstraksie van *S. frutescens* met water, methanol en chloroform.
2. isolering van mikrosomale en mitochondriale P450 ensieme vanuit skaap byniere.
3. inhibisie van die binding van steroied substraat aan P450 ensieme in die teenwoordigheid van *S. frutescens* ekstrakte.
4. effek van *S. frutescens* ekstrakte op die metabolism van progesteroon, deoksikortisol en deoksikortikosteroon in skaap bynier mikrosome en mitochondria
Dedicated to my loving parents Elder & Mrs. O I Egbichi
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The department of Biochemistry, Stellenbosch University for making this study program worthwhile.
Abbreviations

17-OH-PREG  17α-hydroxypregnenolone
17-OH-PROG  17α-hydroxyprogesterone
17β-HSD  17β-hydroxysteroid dehydrogenase
3β HSD  3β-hydroxysteroid dehydrogenase
AAMPS  Association for African Medicinal Plants Standards
ABTS  azinobis-3-ethylbenzothiazoline-6-sulphonic acid
ACTH  adrenocorticotropic hormone
ADX  adrenodoxin
ADXR  adrenodoxin reductase
AP-1  activator protein-1
AVP  arginine vasopressin
BGC  blood glucose concentration
BPH  benign prostatic hyperplasia
BSA  bovine serum albumin
cAMP  adenosine 3’5’-cyclic monophosphate
CNS  central nervous system
CO  carbon monoxide
CORT  corticosterone
COX  cyclooxygenase
CREB  cyclic AMP response element binding
CRH  corticotrophin releasing hormone
CYP11A1  cytochrome P450 side chain cleavage
CYP11B1  cytochrome P450 11β-hydroxylase
CYP11B2  aldosterone synthase
CYP17  cytochrome P450 17α-hydroxylase/17, 20 lyase
CYP19  cytochrome P450 aromatase
<table>
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<tr>
<td>CYP21</td>
<td>cytochrome P450 21-hydroxylase</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detection</td>
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<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOC</td>
<td>deoxycorticosterone</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GABA-BDZ</td>
<td>gamma-aminobutyric acid benzodiazepine</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>HIV</td>
<td>human immuno-deficiency virus</td>
</tr>
<tr>
<td>HPA-axis</td>
<td>hypothalamo-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HS</td>
<td>high spin</td>
</tr>
<tr>
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<td>hydroxysteroid dehydrogenases</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IPP</td>
<td>isopentenyl diphosphate</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>LC-NE</td>
<td>locus coeruleus-norepineprine</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoproteins</td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography/ mass spectrometry</td>
</tr>
<tr>
<td>LS</td>
<td>low spin</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NADP (H)</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>Abbreviation</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PREG</td>
<td>pregnenolone</td>
</tr>
<tr>
<td>PROG</td>
<td>progesterone</td>
</tr>
<tr>
<td>SCC</td>
<td>side chain cleavage</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TEAC</td>
<td>trolox equivalent anti-oxidant capacity</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor -α</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER ONE

Introduction

*Sutherlandia frutescens* (*S. frutescens*), sub-species *microphylla*, is a member of the Fabacea family and is used as a herbal remedy for the treatment of several ailments which include influenza, diabetes, cancer, tuberculosis, chronic fatigue syndrome, rheumatoid arthritis, anxiety, clinical depression, and more recently, those living with human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS) (1-4). Many of the symptoms of these ailments are associated with a perturbation of the stress response which may be associated with disorders of the endocrine system. Of all the traditional plants in South Africa, *S. frutescens* is regarded the most profound in that it is a multipurpose traditional remedy. The plant has enjoyed a long history of use and reports indicating its efficacy as a safe treatment for various health conditions have added to the popularity of this medicinal plant. The extracts of *S. frutescens* have been shown to exhibit anti-proliferative effects on cancer cells, antioxidant activity, and to possess anti-diabetic and anti-inflammatory potential (5, 6), providing scientific evidence for its therapeutic use in the treatment of cancer and diabetes. However, this study focuses on the potential use of this medicinal plant in the treatment of stress and stress related diseases. Chronic stress is characterized by elevated plasma levels of glucocorticoids. These steroid hormones are synthesized in the adrenal cortex in a series of reactions involving the steroidogenic enzymes.

The major aim of this thesis was the determination of the influence of *S. frutescens* extracts on the adrenal cytochrome P450 enzymes. Aqueous, methanol and chloroform *S. frutescens* extracts were prepared and the interaction with the cytochrome P450 enzymes was investigated. The effect of these extracts towards progesterone (PROG), deoxycortisol and deoxycorticosterone (DOC) binding to the cytochrome P450 enzymes as well as their influence on the metabolism of these steroid substrates was investigated. A similar study (7) showed that compounds from the *S. frutescens* extracts could interact with these enzymes and possibly affect adrenal steroidogenesis. This study further investigates the bioactive properties of the plant material in terms of the influence of *S. frutescens* on the cytochrome P450 enzymes and the effect of the manufacturing process on the bioactivity of the plant.

An introduction to *S. frutescens* with reference to its historical background, description and distribution is presented in chapter two. The beneficial medicinal effects of therapeutic plants
typically result from a combination of secondary metabolites present in them. The medicinal actions of plants are unique to a particular plant species or group, and thus combinations of secondary metabolites in a particular plant are often taxonomically distinct (8). The bioactive compounds identified in *S. frutescens* to date include L-canavanine, gamma-aminobutyric acid, and D-pinitol (3). Flavonoids and triterpenoids have also been identified in *S. frutescens* and may contribute towards its therapeutic potential. A review of several recent studies with regard to the anti-proliferative, anti-diabetic and anti-inflammatory properties will be discussed. Safety and efficacy issues that relate to the use of herbal remedies and medicinal plant will also be presented in this chapter.

In chapter three, the P450 enzymes involved in adrenal steroidogenesis will be addressed. This chapter will also discuss the regulation of the hypothalamic-pituitary-adrenal axis and the stress response. Stress can be defined as a change in homeostasis (9) and the response is controlled by the central nervous system (CNS) and peripheral components. Corticotropin-Releasing Factor (CRH) is the principal hypothalamic regulator of the pituitary-adrenal axis, stimulating the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. ACTH in turn stimulates the secretion of glucocorticoid hormones, corticosterone and cortisol, by the adrenal gland (10). Glucocorticoids function in the regulation of the basal activity of the HPA axis and are also responsible for the termination of the stress response by acting on the extrahypothalamic centers, the hypothalamus and the pituitary gland. Chronic activation of the stress system, can lead to several disorders which are the result of elevated CRH secretion with a concomitant increase in glucocorticoid secretion (11).

Adrenal steroid biosynthesis is catalyzed by P450 enzymes and these enzymes are responsible for the monooxygenation and hydroxylation of steroid substrates (12, 13). These membrane bound hemeproteins are monooxygenases requiring molecular oxygen and the coenzyme NADPH. These enzymes, when bound to carbon monoxide and reduced by dithioniate, show a characteristic Soret peak at 450 nm, hence the name P450. The P450 enzymes are classified based on their redox partners (14). Class I P450 enzymes are found in the mitochondrial membrane and are associated with an iron-sulfur protein. This group of enzymes receive electrons from NADPH via adrenodoxin (ADX) and NADPH-adrenodoxin reductase (ADXR) whereas class II P450 enzymes found in the endoplasmic reticulum, need a single redox partner, NADPH-cytochrome P450 reductase (15). This study will focus on three P450 enzymes which includes the cytochrome P450 17α-hydroxylase/17, 20 lyase (CYP17) cytochrome P450 21-hydroxylase (CYP21) and cytochrome P450 11β-hydroxylase
(CYP11B1). In the human adrenal, the microsomal CYP17 catalyzes the conversion of progesterone (PROG) to 17α-hydroxy progesterone (17-OH-PROG) and subsequently to androstenedione. CYP21 catalyzes the conversion of PROG and 17-OH-PROG to DOC and deoxycortisol respectively, while the mitochondrial CYP11B1 catalyses the conversion of DOC and deoxycortisol to corticosterone and cortisol, respectively. Manipulation of these enzymes is of therapeutic importance in the treatment of disease conditions related to increased levels of plasma glucocorticoids found in clinical conditions such as Cushing disease.

Chapter four presents a detailed account of an investigation into the bioactivity of S. frutescens. The interaction of chloroform, aqueous, methanol extracts of S. frutescens as well as S. frutescens tablet extracts, with the steroidogenic P450 enzyme was investigated. Assays were performed using microsomal and mitochondrial enzyme preparation from fresh ovine adrenals. Due to the unique spectral properties of the P450 enzymes, spectral binding assays were performed to determine whether the plant extracts were able to bind directly to the enzymes. The inhibition of the binding of the endogenous steroids by S. frutescens extracts was subsequently investigated. In addition the influence of S. frutescens on the catalytic properties of CYP17, CYP21 and CYP11B1 was determined. PROG, DOC and deoxycortisol metabolism was investigated in the presence of the various S. frutescens extracts.

In conclusion, chapter five presents a summary of the results obtained in this study. Deductions from the spectral binding and steroid conversion assays are used to correlate the bioactivity of S. frutescens to the beneficial properties of this medicinal plant. Results obtained in this study are compared to those previously published in literature.
CHAPTER TWO
Sutherlandia frutescens

2.1 Introduction

Most of the modern medicines have their origins in plants that were often used in the treatment of illness and disease. Invariably, plants and their derivatives contribute to more than fifty percent of all medicine used worldwide. Without plants, most medicine prescribed now would not exist. In Africa there are over 500 species of medicinal plants that have been reported to date. Medicinal plants are not only vital for curing diseases but are a potential source of income to the community. In spite of scientific advances made by modern medicine, 75-80% of the world’s population turns to traditional medicine for healthcare with a rising increase in the interest and use of medicinal plant products being witnessed.

Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary healthcare of poor communities with local therapy being the only means of medical treatment for such communities. However, some side effects which may arise by the use of traditional medicine could be due to irregularities such as adulterated or inadequate research of the herbal plant (16).

Despite scant evidence on the effectiveness and safety of medicinal plants, health ministries of several African nations have recommended traditional medicines for treating HIV/AIDS and its related diseases. It has therefore become necessary to validate medicinal claims with scientific research and clinical studies to establish the safety and efficacy of traditional medicine

Indigenous medicinal plants are used by more than 60% of South Africans for health care needs or cultural practices (17). In South Africa, as in most developing countries of the world, traditional herbal medicine still forms the backbone of rural health care. Approximately 3 000 plant species are used by an estimated 200 000 indigenous traditional healers (18). Amongst these numerous plants is S. frutescens, on which this thesis will focus.
*Sutherlandia frutescens* R.BR. (Fabaceae) which belongs to the class *Magnoliopsida* and the order *Fabales*, is one of South Africa’s medicinal plants traditionally used for the treatment of several ailments. It is used as an internal medication for treating stomach problems, fever and backache and can be used topically in treating wounds and eyes infections (19). The plants extracts have been shown to exhibit an anti-proliferative effect on cancer cells (6). *S. frutescens* is considered as a safe medicinal plant for the treatment of several ailments such as tuberculosis, fatigue, peptic ulcers, gastritis and anxiety.

The Fabaceae family contains 600 genera and 1200 species, distributed throughout the world. The genus *Sutherlandia* was named after James Sutherland and the species, *frutescens*, means bushy in Latin. Other related species of *Sutherlandia* includes; *S. microphylla, S. montana* (mountain cancer bush) and *S. tomentosa*. The *Sutherlandia* species cannot be easily differentiated as they closely resemble each other. These species are unevenly distributed in the Western Cape Province in South Africa and are also found in Botswana and Namibia (Figure 1). This family of plants is represented by 134 genera and more than 1300 species. *S. frutescens* comprises of six taxa and is common in South Africa (20). 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.

![Figure 1. The shaded area, marks the geographical distribution of *S. frutescens* in the Western, Eastern and Northern Cape provinces of South Africa.](image)

The medicinal application of *S. frutescens* originated from the Khoi and Nama people. They used decoctions to treat fevers, wash wounds and for a variety of other ailments. There are different dialects in South Africa and each one describes the plant differently. The traditional Tswana name given to *S. frutescens* is Phetola, which is in accordance to the favorable outcome when used in treating an illness. The Northern Sotho name for *S. frutescens* is Lerumo-lamadi, (spear for the blood) and refers to its use as a blood-purifier or an all-purpose
tonic. The Zulu name, Insiswa, means that it dispels darkness and is used as an anti-depressant and as a calming tea. It is called Kankerbos in Afrikaans and used in the treatment of cancer and as an anti-diabetic treatment (21).

*Sutherlandia frutescens* has soft, saw-like edged leaves which are hairy on the surface and have a silvery appearance (22). The plant produces red flowers from July to December. Its fruit is an inflated leathery pod that is 1.3 – 2 mm long. It is a robust, 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. Cultivation of *S. frutescens* is usually done on a large scale in autumn or spring and germination occurs 2-3 weeks later.

![Figure 2 Flowering S. frutescens found on Table Mountain, Western Cape, South Africa.](image)

Commercially available *S. frutescens* is produced from organically cultivated plant material (23). During harvesting, only leaves and sometimes tender stems are selected. After the harvesting of *S. frutescens*, the selected leaves and tender stems are dried at a temperature of 40 °C or directly under sunlight for a few days. The flowers, pods and seeds are discarded. The dry product is stored under controlled conditions (dry, hygienic and ventilated) before its processing. During processing, the dried leaves and tender stems are ground into powder using a suitable mill. The powder is subsequently sieved, removing any hard pieces of dried stems.

*Sutherlandia frutescens* is commercially available as capsules that contain the raw plant material in powdered form. These capsules are gamma irradiated to achieve microbiological
stability. Phyto Nova, a pharmaceutical company in South Africa is the major distributor of both the powdered and capsulated plant material. The recommended therapeutic dosage of the plant material is 9 mg/kg/day (24).

2.2 Phytomedicine and common medicinal plants used in Africa

The majority of people in developing countries still depend on phytomedicine for their medical healthcare needs. Phytomedicine may be defined as drugs isolated from medicinal plant material. They affect various biochemical pathways, restoring physiological equilibrium and balance. Most of the research on medicinal plant has primarily been focused on the area of phytochemistry. The plants are characterized by identifying various bioactive compounds in them. Medicinal plants may have different bioactive compounds and may thus have side effects.

Regulation of herbal remedy and phytomedicine include quality and safety aspects. In the USA medicinal herbs are marketed as dietary supplements and medical claims are not permitted. In Europe, medicinal plant products are legally regulated and can be registered as traditional phytomedicine. Medicinal plants are subjected to standardization, stability and quality control assessment in order to ensure a reproducible product that will be accounted for batch after batch. Presently in Africa, an organization known as Association for African Medicinal Plants Standards (AAMPS) with its headquarters in Mauritius, has been establishing standards for most of the commercialized medicinal plants as well as others used for long term purposes.

There has been an increase in the interest and use of medicinal plants worldwide as seen by the availability of medicinal plants and plant products at retail outlets, the degree of media coverage and the recent manufacturing of phytomedical products by several major pharmaceutical companies (23). The use of medicinal plants for health care was previously regarded as primitive and unconventional. These plants now play a key role in world health care, with a record of about 80% of Africans depending on them (25) for the treatment of several diseases (26). Studies conducted by the World Health Organization (WHO) in Africa, shows that at least 60% of infants with diseases such as malaria and fever are treated at home with medicinal plants as they are easily accessible (27). Some doctors in Asian countries, such as Japan, prescribe medicinal plants for their patients (28). In first world countries, an
estimated 1500 medicinal plants are used for primary healthcare. The increase in the demand for medicinal plants has led to an increased economic impact on the countries involved in supplying and distributing as several pharmaceuticals companies are now involved in the processing and sales of these plants. The WHO has recorded an estimated annual gross profit of 60 billion US dollars by pharmaceutical companies worldwide. An example of a marketed medicinal plant is Hypericum perforatum, with increased sales of over 20,000% since 1997 (29). Another medicinal plant, Buchu (Agathosma spp.), is well known world wide and large quantities of this plant is exported to food and ornamental industries.

In South Africa, an estimated 3000 medicinal plants are used by traditional healers. A well known South African medicinal plant, Pittosporum viridiflorum, commonly known as cheesewood is used in a variety of ailments. It is found in the Western Cape but also occurs in tropical regions of Africa. The stem bark of Cheesewood is used in the preparation of decoctions for the treatment of abdominal pain, fever, stomach ailments and also in the treatment of cancer.

Another widely used indigenous medicinal plant is Centella asiatica (C. asiatica). The plant is found in riverside areas and is administered as tonics by traditional healers for the improvement of mental function, treatment of depression and could be used as an anti-stress agent (30). C. asiatica has been shown to have antibiotic properties and as such is used for treating leprosy, skin tuberculosis and wounds (31). The plant material is also used to prevent scar formation and is added to cosmetics and skin care products. Bioactive compounds identified in extracts of C. asiatica are the triterpenoid saponins and sapogenins (32).

Most traditional plant medicines are prepared using the leaves or stems of the crude plant material. However, Xysmalobium undulatum, commonly known as Uzara, is one plant of which roots are used for the treatment of diarrhea and dysentery. This root plant can be administered orally when it is prepared with mixture of ethanol and water or can be sniffed when the dry roots are prepared as granules. Uzara is also used for treating hysteria in women and for headaches (33).

Maize (Zea mays), a common plant in Africa, is used to treat urinary infections and cystitis. It helps to reduce frequent urination caused by irritation of the bladder and urethral walls as well
as alleviating the pain in passing urine. It improves urine excretion by soothing and relaxing the lining of the urinary tubules and bladder, thus relieving irritation (34).

*Bidens pilosa*, commonly known as cosmos, is another plant that is commonly found in South Africa that originates from Central America and the West Indies. This plant has a long history of use as a traditional herbal medicine with its leaf preparations being used in the treatment of cancer (35). Asteraceae are also very popular in several African countries such as Nigeria, Cameroon and Ivory Coast and is used to treat several diseases which include malaria and urinary tract infections. Bioactive compounds identified in Asteraceae leaf extracts include terpenoids and glycosides (36). These compounds have been shown to possess an anti-bacterial, anti-malaria and anti-tumor activity (35). Pyrethroids are also extracted from them and are used as insecticides.

In comparison to synthetic drugs, medicinal plants are less expensive and have great value in export trade and pharmaceutical industries. Their therapeutic benefit includes their wide range usage in the treatment of several ailments, including chronic treatments. The effects of medicinal plants typically result from various secondary metabolites and the anti-microbial, anti-inflammatory, anti-bacterial and anti-stress activities are attributed to these metabolites.

### 2.3 Secondary Plant Metabolites

There are several chemical compounds which can be found in the cells of plants. These compounds may be divided into primary and secondary plant metabolites. The primary plant metabolites include carbohydrates, fats and proteins. Secondary plant metabolites include organic compounds, and are generally regarded as not having a direct role in plant growth and development. They are produced by pathways derived from primary metabolic pathways. Although the term secondary metabolite could present these compounds as a type of plant by-product, they are nevertheless essential as they play a major role in the survival of the plant. A typical example is seen by curcumin, a compound which protects the Curcuma plant rhizomes from diseases. Another example is seen with shikimic acid, a compound found in plants such as *Illicium religiosum*, which plays a major role in the biosynthesis of aromatic compounds (37).
Many bioactive compounds have been discovered and many drugs prescribed today are derived from secondary plant metabolites. These compounds vary widely in chemical structure and function and are classified accordingly. A list of these secondary plant products includes compounds such as terpenoids, flavonoids, phenolics, polyisoprenes, cynogenic glycosides, carotenoids and alkaloids. Although these secondary plant products are common, they are, however, plant species specific.

Most of the phenolic compounds in plants belong to the flavonoid group. The flavonoids are mostly found in fruits, vegetables and extracts obtained from plants. As such, they are natural dietary disease-preventing, health-promoting, anti-ageing substances (2). Flavonoids and some of the aromatic amino-acids such as phenylalanine, tryptophan and tyrosine are synthesised via the shikimic pathway in the plastids of plants which may be categorized in three different steps. The first step is the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate, which leads to the formation of shikimic acid. The shikimic acid is converted to chorismate, in a number of enzyme catalyzed reactions. Chorismate is subsequently converted to several products which includes the flavonoids (38, 39 and 40). The different types of flavonoids (Figure 3) are classified based on the general structure derived from the C15 flavan ring system. Some of the classes include flavonols, flavanones, flavones, flavanols, isoflavone and anthocyanidins.

Figure 3. Flavonoids identified in medicinal plants.
Some of the major types of flavonols such as quercetin are found in plants such as *Ginkgo biloba*. Flavonoids have also been identified in *S. frutescens*. These compounds are some of the most effective anti-oxidant compounds available to humans. They exert their anti-oxidant effects by neutralizing or by chelating different types of oxidizing radicals (41) which includes the superoxide (42) and hydroxyl radicals (43).

Pinitol is a naturally occurring compound that is synthesized in the leaves of legumes and can also be found in food such as soy. This mono-methylated form of D-chiro-inositol (Figure 4) has also been isolated from *S. frutescens* leaves, and is used for treating muscle wasting in cancer and AIDS patients (44).

![Figure 4. Pinitol.](image)

Pinitol has also been used for the treatment of diseases related to insulin resistance such as obesity, hypertension and diabetes mellitus (45). The presence of pinitol is thought to be one of the contributory factors of the anti-inflammatory activity shown by *S. frutescens* extract against acute edema in rat models. *S. frutescens* was shown to reduce the production of pro-inflammatory cytokines: tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) (46).

Another group of natural products found in plants are the terpenoids. This group of compounds constitutes the highest class of natural plant products in plants. Terpenoids are found in fungi and in insects such as termites (47). The biosynthesis of the various types of terpenoids, which includes monoterpenoids, diterpenoids, triterpenoids and sesquiterpenoids, starts with mevalonic acid as the precursor molecule. Upon phosphorylation this acid is converted into a phosphorylated isoprene and is then polymerized. Most of these terpenoids are of therapeutic importance.
Menthol, an example of the monoterpenoids consisting of 10 carbon atoms, is used as a pain reliever. An example of a diterpenoids is taxol which was first isolated from the plant *Taxus brevifolia* and has been used therapeutically in the treatment of cancer. The triterpenoids are the most abundant form of the terpenoid group. These compounds are usually glycosilated with the largest of this class being the oleanane group. These compounds include limulatone, which serves as a defence substance for the plant *Collisella limulata*; arbruside E, a compound isolated from *Arbrus precatorius*, used therapeutically as a purgative; and bruceantin isolated from *Bracea antidyssenterica*, which is used for the treatment of cancer (Figure 5) (48).

![Figure 5. Triterpenoids isolated from *Arbrus precatorius* (A) Arbruside E and *Bracea antidyssenterica* (B) Bruceantin](image)

The triterpenoid commonly known as SU1 and SU2 have been identified in *S. frutescens* (Figure 6). These compounds are known to have some biological activities and are commonly used against bacteria, fungi and viruses (49, 50 and 51) however the mechanism of action of these compounds is unknown.

![Figure 6. Novel triterpenoids identified in *S. frutescens*.](image)
Studies have been carried out to investigate the immune-stimulating, anti-inflammatory and anti-microbial properties of these compounds (52, 53, 54 and 55). Results obtained from a study on the anti-viral activity of the triterpenoid saponins indicate that these compounds could interfere with the virus replicative cycle within the cell (56) which may thus support the use of this plant material in the treatment of HIV/AIDS.

Another group of natural products found in plants are the alkaloids. These are heterocyclic nitrogen containing compounds originating from amino acids. Different types of alkaloids have been identified in species from over 300 plant families (56). These compounds are classified based on their common molecular precursors. These include indole, pyrrolidine, phenethylamine, purine, isoquinoline, and quinoline molecules. Alkaloids such as morphine and codeine are used therapeutically as pain relievers. Morphine was first isolated from the opium poppy *Papaver somniferum* and was used as an anesthetic (57). Other plants in which these alkaloids have been identified include *Solamun khasiamum, Sceletium tortuosum* and *Cinchonac succirubra*. A microchemical assay done on *S. frutescens* revealed that there are no alkaloids in the plant material. However, gama amino butyric acid (GABA), which accumulates in most plants tissues as a result from adaptation during heat stress, has been identified in the plant (58). GABA can also be found accumulated in plant cells once they are exposed to either biotic or abiotic stimuli such as cold-shock, darkness, drought or salinity.

![Figure 7. Gamma amino butyric acid.](image)

In plants, GABA functions as a signaling molecule and can alter metabolism, growth and development. In the human body, it functions as an inhibitory neuro-transmitter in the central nervous system as well as a modulator of brain dopamine. GABA is found at levels of 14 mg per gram in *S. frutescens* dried leaf. An in-vitro study on the anti-proliferative effect of GABA in *S. frutescens* showed that this bioactive compound can inhibit the migration of tumor cells (59). The potential activity of GABA, against the pathophysiology of anxiety and depression has also been reported (60, 61).
A non-protein amino-acid found in plants is L-canavanine, an arginine analogue with documented anti-viral, anti-bacterial, anti-fungal and anti-cancer activities. It acts as an L-arginine antagonist and has patented anti-cancer (62) and anti-viral activity, which includes the influenza virus and the retroviruses (63). L-Canavanine (Figure 8) is also a selective inhibitor of inducible nitric oxide synthase and therefore has possible applications in the treatment of septic shock and chronic inflammation (64).

Figure 8. L-Canavanine.

L-canavanine has been identified as one of the major bioactive compounds in *S. frutescens*. There is approximately 30-40 mg per gram dried Sutherlandia leaf (65), whereas *S. frutescens* tablets manufactured by Phyto Nova contain 3 mg of canavanine per gram of tablet (66). Other small amounts of secondary plant metabolites such as methyl parabens, propyl parabens, hexadecanoic acid, gamma sitosterol, sigmast-4-en-3-one, and several long-chain alcohols typically found in plants, are also present in *S. frutescens* (54).

Although L-arginine and asparagines are not secondary plant metabolites, they have been identified in *S. frutescens*. Several clinical studies have been done to validate the therapeutic effect of L-arginine. It is used as an anti-viral, as it is a precursor for the synthesis of nitric oxide (NO) and is used to improve the immune function (44).

### 2.4 Preparation and dosage of medicinal plants

Plants are used in traditional medicine as infusions, decoctions and tinctures. Infusions are preparations in which the plant material is placed in oil or water (without boiling) and strained after 10 minutes. A decoction is a preparation made by adding cold water to the required amount of herb, boiled for 10 minutes and strained. An alcoholic tincture contains 30% of water and 70% alcohol in which the plant material is boiled for a specific time and strained. Extractions prepared with organic solvents such as methanol and ethanol are thought to be of
a more superior preparation compared to other means of preparation. These alcohols easily extract volatile oils and alkaloids from the plant material and can also serve as a storage medium. Hence they are mostly preferred in the preparation of these medicinal plants. In some cases, treatment may also be carried out by direct ingestion of the plant material. As a common practice, the plant material may also be placed in hot or cold water and administered orally.

Consideration is given to the active compounds present in medicinal plants. The use of alcoholic extracts may result in ineffective treatment or harmful side effects compared to the use of traditional water extracts. The concentration of active compounds and other plant metabolites in harvested herbal plants is affected by several factors which include variation in the plant part used, maturity and period of harvesting the plant, topographical condition such as soil acidity, water condition and contaminants in the soil, weather conditions and other growth factors.

Not only is the administration important for the effectiveness of the compounds but so is the correct dosage. The amount administered may vary in the range of 5g–50 g of dry plant material per liter. Higher doses of plant material are seldom used or recommended in traditional medicine. Dose variation has greater effects on children due to their smaller size and their different ability to detoxify chemicals (67).

2.5 Efficacy and safety in traditional medicine

During the past decades, the use of herbal medicine has increased notably not only in developing countries but also in first world countries (68, 69). This has increased the international trade in herbal medicine and has attracted many pharmaceutical companies. It has been reported that, in the United states of America, more than 220 herbal medical companies exist while in Europe, over 2000 such companies have been established (70, 71). Depending on the particular country and existing legislation, herbal products used for diagnosis, cure, mitigation, treatment or prevention of diseases are normally regulated as drugs. A typical example is seen in Germany, where the chemical cynarin extracted from the artichoke plant is sold in tablet or capsule for the treatment of hypertension and liver disorders (72).
However, in some countries, including the United States, botanical products are marketed as “dietary supplements”. For herbal preparations to be classified as drugs, they need to be registered and tested to prove their safety and clinical efficacy. However, to date, few programs have been recognized in order to determine the safety and efficacy of herbal medicines as originally proposed by the World Health Organization (WHO) (73, 74). Most studies on medicinal plants are channeled towards their efficacy and safety during their use in the treatment of several diseases.

Many patients consider medicinal plants are safe since they are natural. However, it is necessary to evaluate and authenticate these plants in order to avoid possible side effects that could result from their use. Studies such as analytical and double-blind clinical trials are required to be performed to determine the safety and efficacy of each medicinal plant prior to their recommendation for therapeutic use (75). Other methods employed in validating the efficacy and safety of medicinal plants includes ethnobotanical claims, anecdotes and observational studies (76).

The majority of the medicinal plants used against infections are effective mainly due to the bioactive compounds the plants produce to protect themselves against pathogens. Invariably, these compounds when extracted, serve as protection against viral, bacterial and fungal infections in humans. However, some of these medicinal plants may contain different bioactive compounds of different complexity and thus could give rise to unwanted side effects. For instance, digitalin, which is extracted from the leaves of *Digitalis purpurea* (foxgloves), is used for the treatment of heart conditions and used to increase cardiac contractility. Side effects such as diarrhea, nausea and wild hallucination brought on by ingestion have been reported. Nevertheless, the adverse effects of most herbal drugs are comparatively less frequent when the drugs are administered correctly in comparison to synthetic drugs (77). For instance the extract of the plant *Hypericum perforatum* (St John’s wart) which is known for its anti-depressant activity, has been found to have a less adverse effect compared to synthetic analogues such as tricyclic anti-depressants or monoamine oxidase inhibitors (78).

It is generally considered that assessing medicinal plant material is not necessary before their use in primary healthcare by the rural communities since they are natural. However, quality control and standardization of medicinal plants is necessary whether the plant is being used as
a primary health-care by rural people or by pharmaceutical companies. The entire process of standardization is required to maintain a safe and effective brand of the plant material. The process involved in the standardization of plant material includes several steps — cultivation, ethno-pharmacology, isolation and identification of bioactive compounds. Other processes include pharmacology, safety, standardization and clinical evaluations.

The source as well as the quality of the raw plant material plays an essential role in ensuring the quality and stability of the marketed herbal product. Factors which affect the quality and therapeutic value of herbal medicines are exposure to light and high temperatures, availability of water and nutrients during growth, time and method of collection, drying, packing, storage and transportation of raw materials, as well as the age and part of the harvested plant. In addition, extraction procedures, contamination with microorganisms, heavy metals and pesticides, can equally affect the quality, safety and efficacy of herbal drugs (79). For the abovementioned reasons, pharmaceutical companies prefer using cultivated plants instead of wild-harvested plants, since they show smaller variation in their constituents. Furthermore, when medicinal plants are produced by cultivation, the main secondary metabolites can be monitored and this permits defining the best period for harvesting (80, 81).

The process of isolation, purification and structure elucidation of bioactive compounds has made it possible to establish appropriate strategies for quality control and for the standardization of herbal preparations. Techniques used for quality control and in the standardization of both the raw material and herbal drugs include TLC, gas chromatography (GC), high-performance liquid chromatography (HPLC), mass spectrometry (MS), liquid chromatography/ mass spectrometry (LC/MS) infrared-spectrometry and ultraviolet/visible spectrometry, used alone or in combination.

2.6 Adverse effects of traditional herbal medicines

There are several contributory factors that could lead to side effects by the use of traditional medicines. This includes the sex and age of the patient, nutritional status and prevalent diseases. Side effects can also arise due to irregularities of production and processing or an over-dose of the herbal preparations (82, 83). Adverse side effects can be classified as intrinsic or extrinsic.
The intrinsic effects are usually effects produced by the herb itself and two types have been characterized, type A and type B. The type A effect is a predictable and dose-dependent effect while the type B effect is an unpredictable and idiosyncratic reaction (84). An example of a medicinal plant with adverse side effects is, *Pausinystalia yohimbe* which contains an alkaloid yohimbine found in the bark. This alkaloid has an $\alpha_2$-adrenoreceptor antagonist activity and is used for male infertility. It causes hypertension and anxiety in a predictable, dose-related manner (type A reaction). The alkaloid is equally associated with the allergic reactions of bronchospasm and increased mucus production when taken in normal doses by patients with severe allergic dermatitis (type B reaction) (85, 86). Other forms of type A reactions include accidental poisoning and deliberate overdose.

The extrinsic effect is due to inappropriate manufacturing procedures. A list of these irregularities includes misidentification of plant material, substitution or adulteration, impurities due to contaminants, lack of standardization, incorrect preparation and inappropriate labeling (87). During storage of the harvested plant material, the plant can be contaminated due to poor safety measures, pests and micro-organisms. Plants are easily spoiled by micro-organism and fungi, and this contributes towards the side effects in herbal medicine (88). The source of these contaminations can be associated with the cultivation or the processing of the plant material. However, boiling the plant material can change the alkaloid composition, reduce the plant’s toxicity and can significantly reduce microorganism contamination (89). However, some heavy metals such as cadmium, lead, mercury, thallium and arsenic have been reported as contaminants of some herbal preparations (90, 91).

The method by which the raw plant material is processed by the manufacturer, the traditional practitioner or patient, determines the pharmacological activity of the final product. Thus, incorrect preparation and dosage contributes to extrinsic side effects. Misidentification of plant material is one of several errors made in the usage of traditional herbal medicines and can be associated with side effects. Plant material can be misidentified during the manufacturing process or while the raw plant is being picked. Most of the plants used for herbal medicine can be named in different ways such as the scientific, latinized, pharmaceutical and the common English name. The naming of the plant may therefore make it difficult for medical practitioners to identify a particular herbal plant correctly and subsequently associate adverse effects with its usage (92). Misidentification of medicinal herbs may inevitably result in misadministration of the material. However, instrumental
analytical techniques such as spectrometric and chromatographic technique can serve to isolate and identify natural products present in plant materials.

Adulteration and substitution of plant material due to scarcity and economic reasons is one of the major factors that contribute to extrinsic side effects. Scarcity of a particular medicinal plant could lead to the substitution with another plant. The quality of herbal medicines may also be compromised by plant popularity, resulting in its adulteration. The substitution of plants can however, also be due to an error in identification. Irrespective of the reason for the adulteration or substitution of any particular plant material, the resulting outcome may result in adverse side effects.

Several cases arising from the use of intended adulterated or substituted medicinal plants have been reported. An example of this is a case where the use of a medicinal plant product known as “Tung Shueh” used for the treatment of arthritis, resulted in acute interstitial nephritis, loss of blood pressure control, peptic ulceration and reversible renal failure. This was as a result of the presence of compounds such as diazepam and mefanamic acid, which were not listed on the product label (93, 94).

One way to ensure safety and efficacy of traditional medicine is through regulation and legislation of the medicinal plants. The legal procedure for regulating and legislating herbal medicines varies from country to country. This is due to the different cultural beliefs and that herbal medicines are rarely studied scientifically. Thus, few herbal preparations have been tested for safety and efficacy. The WHO has published guidelines in order to identify important procedures for assessing quality, safety, and efficacy of herbal medicines, which is aimed at assisting national regulatory authorities, scientific organizations and manufacturers in this particular area (95). In addition, the WHO has prepared pharmacopeic monographs on traditional medicines and the basis of guidelines for the assessment of herbal drugs (96, 97). A detailed guideline describing the norms and guidelines, regulatory models for herbal medicines and its selection and prescription as an alternate medicine or dietary supplement currently exist (98).
2.7 Toxicity

The toxicity of most medicinal plants could arise as a result of their interaction with other plants or drugs, their natural toxicity or due to the active compounds present in them. Toxicity may also be due to contaminants, substitution or adulterants.

*Sutherlandia frutescens* has a comparatively long history of seemingly safe usage in South Africa. However, a few side effects may include occasional mild diarrhea, dry mouth, mild diuresis, and dizziness (65). In an effort to ascertain the safety in the use of *S. frutescens*, an extensive toxicology screening was carried-out in a primate model using a higher dosage than the recommended dose of 9 mg/kg/day. Results showed *S. frutescens* did not to exhibit clinical or physiologic toxicity (24).

Although long-term studies of *S. frutescens* extracts have not been documented, results indicating negative effects of the plant material have not been reported.

2.8 Bioactive properties of *S. frutescens* extracts

*Sutherlandia frutescens* is a well established medicinal plant used in the treatment of several diseases. This plant has long been known to exhibit biological activity against bacteria, fungi and virus and as such has been used in anti-bacterial and anti-viral treatments. Since *S. frutescens* has many therapeutic effects, it has been the subject of several investigations. *S. frutescens* contains several biological active compounds which include pinitol, GABA, L-arginine and several other compounds that influence the human physiology (66). It is probable that these compounds in *S. frutescens* act synergistically mediating a greater clinical effect than that previously obtained from a particular single compound. Several investigations into the anti-cancer, anti-inflammatory, anti-diabetic and hypoglycemic, anti-viral, anti-stress, mutagenicity and anti-mutagenicity, anti-oxidant and anti-convulsant properties of *S. frutescens* have been documented and will be reviewed in this section.

One of the major malignant diseases that affect people is cancer, an ailment which arises due to uncontrolled growth of unwanted cells. Treatment of this disease is usually by the
combination of radiotherapy, surgery and chemotherapy. *S. frutescens* has been used for the treatment of cancer without the undesirable side effects and thus the testing of plant material *in vitro* using various cancer cell lines have been performed in order to understand the mechanism of action and efficiency.

Tai and co-workers reported the anti-proliferative potential of *S. frutescens* in an *in vitro* study using breast cancer (MDA-MB-468) and leukemia (Jurkat and HL60) cell lines (66). Upon comparison with the cells treated with camptothecin and paclitaxel, *S. frutescens* aqueous extracts were found to kill the tumor cells with a higher potency. A cell free azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging assay was applied to evaluate the anti-oxidant activity of the *S. frutescens* extract. Results revealed that low concentrations of *S. frutescens* ethanolic extracts resulted in a hydroxyl radical scavenging activity equivalent to 10 µM of Trolox, as determined in a Trolox equivalent anti-oxidant capacity (TEAC) assay. In addition, *S. frutescens* extracts were shown to mediate potent inhibition of NO production in lipopolysaccharide-stimulated RAW 264.7 cells – a macrophage-like cell line derived from tumors induced in male mice by Abelson murine leukemia virus (66).

One major approach employed in the treatment of cancer is the induction of apoptosis — a form of programmed cell death (99). This is a physiological mechanism that leads to a characteristic cell morphology and death. The morphological changes include blebbing, changes to the cell membranes such as cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation (100, 101). Using methods such as flow-cytometry analysis and Apo percentage™ assay, Chinkwo KA (102) conducted a study to determine if *S. frutescens* extracts could induce apoptosis in cultured carcinoma cells. The Chinese Hamster ovary cells (CHO) and cervical carcinoma (Caski) cells which were used in this study, were treated with *S. frutescens* extracts concentrations ranging from 1.5 and 10 mg/ml, while the control experiment remained untreated. Cell morphology showed cell shrinkage, disintegration and a reduction in cell number. The result obtained from this study and further confirmatory tests such as the dose response and time course experiments indicate that *S. frutescens* extracts could induce apoptosis in the different cell lines (102). A more recent study by Stander et al. (103), further confirms the ability of *S. frutescens* extract to induce apoptosis, particularly in MCF-7 human breast adenocarcinoma cell lines. The cells were cultured and exposed to varying concentration of *S. frutescens* ethanol extract. Results
indicated that 1.5 mg/ml of *S. frutescens* could inhibit 50% of MCF-7 cell proliferation after 24 hours.

Natural or synthetic substances are often employed in chemopreventive approaches in the treatment of cancer to block, reverse or retard the process of carcinogenesis. One such process is the inhibition of the expression or activity of cyclooxygenase (COX)-2, a rate limiting enzyme involved in the inflammatory process. There have been some suggestions that inflammation is closely associated with carcinogenesis and abnormal up-regulation of COX-2 have been observed in several malignancies including those of the breast and urinary bladder tissue and several other organs (104, 105).

A study was conducted by Kundu *et al.* (106) in order to determine if *S. frutescens* methanolic extracts could inhibit the expression of COX-2 in mouse skin stimulated with a tumor promoter. Pathogen free mice were shaved and treated with *S. frutescens* methanolic extracts between 100-200 µg 30 mins prior to the stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA), a prototype tumor promoter (107). Results obtained from this study indicated that the *S. frutescens* extracts had an inhibitory effect on TPA induced COX-2 expression in mouse skin. Hence, this study serves as yet another possible affirmation for the use of the plant in alleviating inflammation as well as *S. frutescens* acting as a chemopreventive agent.

*Sutherlandia frutescens* has also been evaluated for possible mutagenic and anti-mutagenic effects (108). This evaluation is necessary since medicinal plants that indicate anti-mutagenicity have the ability to act as either anticarcinogens or chemopreventive agents. In this study, *S. frutescens* ethyl acetate and methanol extracts were screened for mutagenicity and anti-mutagenic activity in an assay using different bacterial strains of *Salmonella typhimurium*. The result stated in this study shows that *S. frutescens* ethyl acetate extracts had an anti-mutagenic activity while the methanol extract exhibited both anti-mutagenic and promutagenic effects.

A similar study was done earlier by Reid *et al.* (109), where *S. frutescens* and 42 different South African medicinal plants were screened for mutagenic and anti-mutagenic effects using the Ames test. Results obtained showed that only 6 plants, including *S. frutescens* exhibited anti-mutagenicity.
A study that examined the anti-inflammatory effects of aqueous extracts of *S. frutescens* shoots in experimental models of edema was performed by Ojewole J.A.O. (110). Wistar rats were divided into three groups; a control, and groups treated with either diclofenac or *S. frutescens* extract. Diclofenac is an anti-inflammatory drug that is also used as an analgesic and it has been suggested to be useful in the treatment of urinary tract infections caused by *E. coli* (111). For the rat hind paw edema, acute inflammation was induced by sub-plantar injection of a phlogistic agent (fresh egg albumin). These are vehicles that provide a skin inflammation model suitable for analyzing topical anti-inflammatory agents. The paws were monitored and an increase in the linear diameter of the point where the phlogistic agent was administrated served as an indication of inflammation. Assessment showed that sub-plantar injection of fresh egg albumin led to an increase in the hand paw diameters of the control and untreated rats. However, there was a significant reduction in the induced acute inflammation of the rats treated with *S. frutescens* aqueous extract. This result is an indication of anti-inflammatory activity by the plant material (112). It is possible that the mechanism of action of diclofenac in exerting its anti-inflammatory activity such as inhibiting the release, synthesis and production of inflammatory mediators, may also be applicable to *S. frutescens*.

One of the studies that evaluated the anti-oxidant effect of *S. frutescens* was conducted by Fernandes *et al.* (2). The different studies shown above, presented results which justified the therapeutic usage of *S. frutescens* for the treatment of internal cancer and inflammation. This ability of *S. frutescens* to show an anti-inflammatory and various medicinal effects is thought to be partly due to their anti-oxidant activity (113, 114). This beneficial effect of *S. frutescens* anti-oxidants, concentrates on its protection against oxidative damage caused by reactive oxygen specie (ROS) (115). An example of these reactive oxygen intermediates that are responsible for the pathogenesis of several inflammatory condition are the neutrophil derived oxygen intermediates; superoxide radicals. Hence this study investigated the effects of *S. frutescens* aqueous extracts on luminal and lucigenin enhanced chemiluminescence by L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-stimulated neutrophils.

The neutrophils were incubated in either luminal or lucigenin for a time period (30 mins) and then added to different concentration of *S. frutescens* extracts. The neutrophils were then activated by the addition of FMLP and the rate of oxidant production was subsequently monitored and measured as emitted chemiluminescence. The result provided, showed that *S. frutescens* aqueous could decrease the lucigenin and luminal enhanced chemiluminescence.
response of neutrophils stimulated by FMLP in a dose related manner. This confirms an anti-
oxidant potential in the plant material, which however could be due to bioactive compounds such as the flavonoids or phenolic compounds.

Diabetes is one of the most prevalent diseases in the world. Several studies have been done investigating the anti-diabetic action of *S. frutescens*. In a study where streptozotocin (STZ)-induced diabetic rats (110), was examined, the result showed that *S. frutescens* could induce a significant hypoglycaemic effect in STZ-treated rats. This result is comparative to the outcome of a treatment obtained with 250 mg/kg of chlorpropamide. Chlorpropamide is a hypoglycaemic agent used in the treatment of type-2 diabetes. Results obtained from this study show that the maximum reduction of the blood glucose concentration (BGC) in normoglycemic rats was 25.43 % and 34.98 % upon treatment of the animals with 800 mg/kg of *S. frutescens* extracts and 250 mg/kg of chlorpropamide, respectively. However, STZ-induced diabetic rats treated with *S. frutescens* extracts were able to maintain their reduced BGC levels longer than those treated with chlorpropamide (110). It is thus justifiable to suggest that the hypoglycemic effect of the plant material is shown in a mechanism similar to that of chlorpropamide.

In a related study by Chadwick *et al.* (5), the anti-diabetic effects of *S. frutescens* leaves was evaluated and compared with metformin in rats rendered hyperinsulinaemic. Metformin is one of the anti-diabetic drugs from the Biguanides class, used for the treatment of type 2 diabetes. Wistar rats were fed with diet that induced obesity, insulin resistance and were rendered to a pre-diabetic state. The rats were subsequently divided into three groups — a control group, a group treated with metformin and one with *S. frutescens* in their drinking water. No significant weight gain was observed amongst rats treated with *S. frutescens* aqueous extract. In addition, *S. frutescens* promoted the uptake of glucose by muscle and fat tissue, with glucose clearance being similar to that of the metformin group. A decrease in muscle glycogen content as well as an inhibition of intestinal glucose uptake in rats treated with *S. frutescens* aqueous extract was observed. The result obtained in this study confirms *S. frutescens* as a hypoglycaemic agent, hence justifying its use in the treatment of diabetes. It is possible that, in this context of diabetes, the anti-oxidant constituents in *S. frutescens*, which include bioactive compounds such as L-canavanine, saponin and pinitol are responsible for this therapeutic benefit.
Several studies have been carried out to determine the immune boosting properties of *S. frutescens* in HIV/AIDS patients. The use of natural products in the treatment of HIV/AIDS is directed mainly at the ability of medicinal plants to inhibit HIV replication as well as boosting the immune system of the patients. Most HIV-positive patients, while on appropriate therapy together with *S. frutescens*, show an improvement in muscle mass characterized by an increase in weight after a period of six weeks (116). An overall sense of well-being together with an improvement in appetite, sleep, exercise tolerance and a decrease in anxiety has been reported by clinicians in South Africa. However controlled clinical trials need to be done to validate these anecdotal reports on the use of *S. frutescens* in the boosting of the immune status of people living with HIV.

A study was performed by Pascal *et al.* (117) investigating the inhibitory activity of *S. frutescens* extracts, alongside with sixteen different South African medicinal plants, against HIV-1 reverse transcriptase (RT) and integrase (IN). The RT functions in transcribing viral RNA into viral DNA. Inhibition of HIV-1 RT activity was evaluated by measuring the incorporation of methyl-\(^{3}\)H thymidine triphosphate template primer in the absence and presence of the plant extracts. For the HIV-1 RT RNase H activity, a radiolabelled RNA/DNA hybrid was used as a substrate for the RNase H. The result obtained showed that the *S. frutescens* methanol extract stimulated the RT activity.

In a similar study by Harnett *et al.* (118), a screening method was performed using *S. frutescens* extracts. Extracts were prepared and subsequently subjected to ethanol precipitation to determine if the anti-HIV effect was as a result of sulphated polysaccharides. Other strategies employed in this study were reverse transcription (RT) and the glycohydrolase enzyme assay. The inhibition of these enzymes is thought to be effective in reducing the infectivity of the HIV viron (119). Result obtained from this study, showed that *S. frutescens* extracts contain bioactive compounds that could inhibit the glycohydrolase and HIV transcriptase enzymes. In the glycohydrolase enzyme assay, a *S. frutescens* methylene dichloride extract showed a greater inhibitory effect on both the \(\alpha\)- and \(\beta\)-glucosidase enzymes in comparison with *S. frutescens* aqueous extract.

In contrast, the results provided by Pascal *et al.* (117) showed that *S. frutescens* methanol extract stimulated the RT activity. The different effects shown by the plant material in both studies could be as a result of the different experimental procedures. Similarly, other South Africa herbs have been effective in the treatment of HIV/AIDS due to their ability to boost the
immune system and having the ability to inhibit HIV replication, as was found with *S. frutescens* (118). Hence the result obtained from this study could point to a possible mechanism of action of *S. frutescens* in aiding HIV-positive patients.

There have been positive reports on the use of *S. frutescens* in the treatment of stress and stress related diseases (58, 120). Stress could be referred to changes in homeostasis and involves several reactions of the body in response to unfavorable conditions. Elevated blood glucocorticoid levels resulting from stress have been linked to other ailments such as immunosuppression and cardiovascular diseases.

*Sutherlandia frutescens* has traditionally been used for decades to treat the symptoms of stress and illnesses associated with stress which are generally associated with increase plasma glucocorticoid levels (58). Smith and Myburgh (121) investigated the effect of *S. frutescens* on corticosterone and cytokine levels in a rat model subjected to chronic immobilization stress. Wistar rats were separated into four groups of equal mass — the first two groups served as controls either for those that received a placebo (isotonic saline) or *S. frutescens* treatment. The third and fourth groups were subjected to chronic immobilization stress after being treated with either the placebo or *S. frutescens* infusions. Immobilization was performed by placing individual rats into small cages, preventing them from moving freely for 2 hours over a period of 28 days. This method is commonly used to induce mild but physiological significant stress to experimental rats (122, 123). The rats were slaughtered and serum samples were analyzed for corticosterone using a radio-immunoassay. As expected, the result showed that intermittent immobilization of the rats led to a marked increase of the basal serum corticosterone concentration. There was a decrease in the corticosterone response to chronic intermittent immobilization amongst the rats treated with *S. frutescens* extracts. Furthermore in the control rats, the extract was able to increase basal corticosterone concentration more than the placebo-supplemented rat control.

In a related study, Prevoo *et al.* (7) investigated the influence of *S. frutescens* on the cytochrome P450 enzymes which catalyse the biosynthesis of the adrenal steroid hormones. The binding of natural substrates to CYP17 and CYP21, two key enzymes in the biosynthesis of glucocorticoids, were assayed in the presence of extracts and bioactive compounds, L-canavanine, pinitol, GABA, flavonoids and triterpenoid glucosides present in *S. frutescens*. The triterpenoid fraction inhibited both pregnenolone and progesterone binding to the enzymes while aqueous and methanol extracts inhibited progesterone binding only. Pregnenolone and progesterone
metabolism was assayed in the presence of extracts and it was shown that the inhibition of CYP21 was greater than that of CYP17. The influence of the extracts on the biosynthesis of glucocorticoid precursors may be a possible mechanism with which *S. frutescens* is able to reduce glucocorticoid levels and alleviate symptoms associated with stress (7).

A more recent approach towards the evaluation of the biological activities of *S. frutescens* has been channeled towards its anti-convulsant activity. Convulsion, also regarded as seizures, is a disease that is associated with abnormal brain functions. These clinical conditions are characterised by sudden fall to the ground, foaming in the mouth, gnashing of teeth, involuntary muscle contraction, snorting and loss of bladder control. Traditional practitioners in the KwaZulu-Natal province of South Africa use decoctions and infusions of *S. frutescens* in the treatment of childhood convulsion and epilepsy. Ojewole J.A.O. (124) investigated the anti-convulsant properties of *S. frutescens*. Compounds such as pentylenetetrazole (90 mg/kg), picrotoxin (10 mg/kg) and bicuculline (30 mg/kg) were used to induce convulsions in healthy male *Balb C* mice. The mice were separated into three groups — a control group, one treated with the reference anti-convulsant drugs (phenobarbitone 20 mg/kg or diazepam 0.5 mg/kg) and one treated with *S. frutescens* aqueous extract. After the induction with the convulsant agents, the mice were monitored for signs such as hind–limb seizures. Intraperitoneal injections of the various convulsant agents led, as expected, to hind-limb tonic seizures in the mice. However, in mice treated with *S. frutescens*, the extract produced dose related protection against the various convulsant agents. It is possible that the presence of bioactive compounds such as GABA, an inhibitory neurotransmitter, in *S. frutescens* could be responsible for the anti-convulsant properties, justifying the traditional use of *S. frutescens* as an anti-convulsant agent.
2.9 Summary

Medicinal plants have for centuries played an important role in the discovery of new drugs. *S. frutescens* is one of such medicinal plants that have been used for over a hundred years in South Africa. The wide range of therapeutic applications of *S. frutescens* includes its use in the treatment of cancer, diabetes, HIV/AIDS, stress and its related diseases. The use of *S. frutescens* has been boosted by studies showing that there are no side effects or toxicity associated with the therapeutic use of the plant material. Following these claims, many *in vitro* studies and studies using experimental animal models have been performed in order to validate the effectiveness of *S. frutescens*. Major bioactive compounds such as pinitol, triterpenoids, flavonoids, L-canavanine and GABA have been identified in *S. frutescens*. Preliminary scientific research and review literature suggest that these bioactive compounds are responsible for the anti-inflammatory, anti-cancer, anti-viral and anti-diabetic effects in the plant materials.

In the area of HIV/AIDS, the anti-viral effectiveness of *S. frutescens* was established by its ability to inhibit HIV replication. Results from the various *in vitro* studies on the anti-cancer activity of *S. frutescens* showed that the plant material can be used therapeutically for the treatment of cancer since it could induce apoptosis to different cancer cell lines, inhibit NO production as well as inhibiting the release, biosynthesis and production of inflammatory mediators. Further results from recent studies showed the hypoglycaemic activity of *S. frutescens* and as such its usefulness in the treatment of diabetes. The results from experimental animal model studies indicated that *S. frutescens* has anti-stress activity and acts as a potent adaptogen.

Diseases associated with stress have been attributed to the high levels of glucocorticoids. The biosynthesis of glucocorticoids occurs in the adrenal gland in a series of reactions catalyzed by the cytochrome P450 enzymes. However, the catalytic role of these enzymes in steroid hormone biosynthesis will be discussed in the next chapter.
CHAPTER THREE

The cytochrome P450 enzymes involved in adrenal steroidogenesis

3.1 Introduction

Steroid hormones play an important role in physiological functions such as the regulation of fluid and electrolyte balance, sexual development and adaptation to stress condition. These hormones are lipophilic, low molecular weight compounds and are grouped accordingly based on their chemical structure, site of production, biological function and biochemical effects. These steroids are synthesized by endocrine glands such as the adrenals and the gonads and the steroidogenic enzymes present in these catalyse the biosynthesis and metabolism of tissue-specific steroids. This chapter will describe adrenal steroidogenesis, its regulation and will focus on the various cytochrome P450 enzymes.

3.2 Regulation of adrenal steroidogenesis

Adrenal steroidogenesis is strictly under hormonal regulation, mostly by the pituitary trophic hormones, such as adrenocorticotropin (ACTH). The pituitary (hypophysis) is a small tissue mass that lies in a pocket of the sphenoid bone of the brain just below the hypothalamus. It consists of two distinct parts, the anterior pituitary and posterior pituitary, which have distinct embryological origins, functions and control mechanisms (125). The posterior pituitary is formed from neural ectoderm whereas the anterior pituitary originates as an upgrowth from the oral mucosa known as Rathke’s pouch. The anterior pituitary is not directly linked to the hypothalamus, but rather the hypothalamus releases hormone-like substances that directly control the anterior lobe of the pituitary. The anterior pituitary is responsible for the function of most of the endocrine glands but is controlled by the hypothalamus. Under this control, the anterior pituitary secretes hormones such as ACTH and several peptide hormones that regulate physiological processes including stress. The posterior pituitary secretes hormones such as vasopressin and oxytocin which are released into the posterior pituitary capillaries and later into the blood stream (125). Rennin-angiotensin is the primary stimulator of aldosterone synthesis in the glomerulosa, the outer region of the adrenal cortex. The peptide angiotensin II binds to a specific receptor on the surface of the glomerulosa cells (126). The angiotensin II is
produced by the liver and functions in increasing the amount of circulatory mineralocorticoids (127) while ACTH has little effect in the rate of aldosterone secretion.

A decrease in blood Na\(^+\) and blood pressure is detected by the *mascula densa* and juxtaglomerular cells of the nephron (Figure 9). The resulting effect is the secretion of enzyme renin which converts plasma protein angiotensinogen to angiotensin I. Circulatory angiotensin I in the lungs is cleaved by angiotensin converting enzyme (ACE) to form angiotensin II. This subsequently acts on the glomerulosa cells to activate the synthesis of aldosterone.

![Diagram of the renin-angiotensin-aldosterone system](image)

*Figure 9. Schematic representation of the regulation of aldosterone secretion.*

The activation of aldosterone synthesis can also be controlled by factors such as extracellular potassium (128) due to the effect of potassium on the membrane potential of the adrenal glomerulosa cells. The underlying mechanism involves the influx of extracellular Ca\(^+\) through voltage-gated channels (129). Aldosterone synthesis can however be slightly reduced if there is a high Na\(^+\) concentration in the extracellular fluid.

The adrenal glands are involved in the different types of stress condition. The stress response is sub served by the stress system, which has both central nervous system and peripheral
components. Coordination of the stress response is through changes in the activities of the brain and hormonal system known as the hypothalamic-pituitary-adrenal (HPA) axis (9). The central effectors of the stress response are the corticotrophin releasing hormone (CRH) and locus coeruleus-norepineprine (LC-NE)/sympathetic systems. CRH is the major hypothalamic regulator of the pituitary-adrenal axis, which stimulates the secretion of ACTH from the anterior pituitary. ACTH is a 39 amino acid peptide hormone synthesized as part of a larger precursor polypeptide known as proopiomelanocortin (POMC).

During increased periods of physiological or psychological stress and diurnal variation, the hypothalamus secretes CRH, which in turn releases ACTH as shown in Figure 10. ACTH travels through the blood stream and is almost entirely responsible for the control of glucocorticoid production in the adrenals *fasciculata* and *reticularis*. These products will subsequently move to target cells and initiate gene transcription through transcription factors and steroid receptors (SR).

![Figure 10](image_url)

*Figure 10. Schematic representation of glucocorticoid synthesis by adrenal cortex in stress response. Note ACTH and CRH secretion is inhibited by glucocorticoid feedback (130).*

Once the glucocorticoid product such as cortisol is at an adequate or excessive amount, it exhibits a negative feedback, inhibiting the synthesis and release of CRH, ACTH and arginine vasopressin (AVP) from the hypothalamus and ACTH from the anterior pituitary.
AVP is a peptide hormone secreted from the posterior pituitary gland and is released into the brain by different neurons. It is an important releasing factor for ACTH. However, it alone has little effect on ACTH secretion, as it acts in conjunction with the CRH to promote pituitary ACTH release (131) and hypothalamic POMC (132).

AVP and CRH are stimulated by the serotonergic and cholinergic systems of the brain and inhibited by glucocorticoids, ACTH and CRH. Inflammatory cytokines stimulate pituitary ACTH and adrenal cortisol secretion which can lead to high level of these hormones. Regulation of the rate of blood flow is controlled by several neural and hormonal mechanisms. The rate of blood flow through the adrenal gland serves as a regulatory factor in glucocorticoid synthesis (133). This relationship is supported by studies which showed that at conditions such as submaximal ACTH levels, increased blood flow through the adrenals favors the presentation of ACTH to the adrenal gland, which is a major determinant in adrenocortical response (134).

### 3.3 Adrenal steroidogenesis

Numerous organs have the capacity to synthesize biologically active steroids. These include the adrenal gland, testis, ovary, brain, placenta and adipose tissue. For the purpose of this study, this section will discuss the adrenal glands. The adrenal gland lies just above the kidney in humans and most mammals (135). It is the most important steroidogenic tissue in the human body and is essential for survival (136). The adrenal gland is composed of two distinct functional compartments the cortex and the medulla. The cortex and medulla develop from different embryonic tissues and are responsible for the secretion of specific hormones. All steroidogenic processes take place in the adrenal cortex, which is histologically and functionally divided into three concentric zones: the outer glomerulosa, middle fasciculata and reticularis (137). Each zone is responsible for the synthesis of a specific set of steroid hormones. The glomerulosa is responsible for the synthesis of mineralocorticoid and aldosterone, while the reticularis and fasciculata are the source of glucocorticoids (cortisol and corticosterone) and androgens (dehydroepiandrosterone (DHEA) and androstenedione) (138).
The medulla serves as the central core of the adrenal gland and is surrounded by the adrenal cortex. The chromaffin cells of the medulla synthesize and secrete hormones such as adrenalin and noradrenaline. These hormones are part of the fight-or-flight response mediated by the sympathetic nervous system and perform functions such as stimulating the heart-rate, dilation of blood vessel and air passage. The chromaffin cells also secrete along with these hormones several proteins and neuropeptides. These neuropeptides can influence steroid hormone production by stimulating adrenocortical function.

Adrenal steroidogenesis occurs in the mitochondria and endoplasmic reticulum of the adrenal cortex. The biochemical pathway involved in the steroid biosynthesis is similar in all the tissues. However, the difference in the secretory ability depends on the presence or absence of specific enzymes. The steroidogenic enzymes are responsible for the biosynthesis of hormones which include glucocorticoids, mineralocorticoids, progestins, estrogens and androgens. The enzymes include several specific P450 enzymes and hydroxysteroid dehydrogenases (HSDs) and steroid reductases. The mitochondria contains cytochrome P450 side chain cleavage (CYP11A), CYP11B1 and aldosterone synthase (CYP11B2), which are associated with the inner mitochondrial membrane. The endoplasmic reticulum contains CYP21 and CYP17 (139, 140 and 141). Figure 11 represents the pathway of steroid biosynthesis.

![Figure 11. The biosynthetic pathway of steroid hormones in the adrenal cortex and gonads.](image-url)
The precursor of all steroid hormones is cholesterol, a 27-carbon steroid nucleus. The biosynthesis of steroid hormones begins with the conversion of cholesterol to produce the first hormone, pregnenolone (PREG), which is catalyzed by CYP11A1 (142). This reaction is the principal committed, regulated and rate-limiting step in steroid biosynthesis (142). PREG is subsequently converted to progesterone (PROG) by 3β-hydroxysteroid dehydrogenase (3βHSD). The enzyme is widely distributed in steroidogenic and non-steroidogenic tissues and consists of two isoforms type I and type II, which are regulated in a tissue-specific manner (143). PREG can however be hydroxylated to produce 17α-hydroxypregnenolone (17-OH-PREG) by CYP17. This serves as a route for the formation of androgens and estrogens. The formation of androgen in the adrenals is restricted to dehydroepiandrosterone and androstenedione while in the gonads, the presence of the enzyme 17β-HSD, under the control of luteinizing hormone (LH), catalyzes the formation of the male hormone testosterone. CYP17, a single enzyme with both 17-hydroxylase and 17, 20-lyase activities, catalyzes the formation of androgens in the adrenals. These two catalytic activities of this enzyme are due to its distinct catalytic sites (144).

The production of PROG from PREG is dependent on the catalytic activity of CYP17 and 3βHSD. If the catalytic activity of 3βHSD is greater than that of CYP17, PROG is produced through an oxidation and isomerization process (145). The PROG formed can either be hydroxylated at C17 position by CYP17 or at C21 by CYP21 to yield 17α-hydroxyprogesterone (17-OH-PROG) or deoxycorticosterone (DOC), respectively. The 17-OH-PROG produced can be channeled either towards the production of androgens in a reaction catalyzed by the CYP17 or towards the production of cortisol in a two-step enzymatic reactions involving CYP21 and CYP11B1. 17-OH-PROG is converted to deoxycortisol by CYP21. Both DOC and deoxycortisol are transferred into the mitochondria and further hydroxylated at C11 by CYP11B1 to produce corticosterone and cortisol, respectively. DOC may also be hydroxylated at C11 and C18 by CYP11B2, in the final steps of the mineralocorticoid biosynthesis to yield aldosterone.

The CYP11B1, which is expressed in the zona fasciculata and reticularis, has strictly 11-hydroxylase activity alone, whereas CYP11B2 which is restricted to the glomerulosa has additional 18-hydroxylase/aldosterone synthetase activity (146, 147).
3.4 Functions of the adrenal hormones

The various hormones secreted from the adrenals can be categorized into three main groups which include the androgens, mineralocorticoids and glucocorticoids. These hormones have various physiological functions, and are essential in regulating the body homeostasis and metabolism. This section will briefly discuss in general the functions of the hormones which fall into these three categories and their role in the control of the stress response.

3.4.1 Glucocorticoids

The glucocorticoids, corticosterone and cortisol, are produced by the adrenals cortex after its activation by the HPA axis under certain conditions such as stress (148). These hormones regulate several biological processes such as lipid, carbohydrate and protein metabolism (149). Cortisol, which is a more potent glucocorticoid with approximately 95% of all glucocorticoid activity, performs functions such as increasing the enzymes required for the conversion of amino-acids to glucose in liver cells, reducing the rate of glucose utilization in the body, stimulating fat breakdown in adipose tissue and increasing the rate of gluconeogenesis (150). In protein metabolism, cortisol functions in decreasing protein biosynthesis and in the degradation of proteins in muscle cells and also stimulate the expression of liver enzymes involved in amino-acid oxidation (151) Cortisol and other glucocorticoids also show profound influences on several physiologic functions due to their different functions in growth, development and maintenance of basal and stress-related homeostasis (152). Cortisol also functions in reducing allergic response and suppresses the immune system. When a stressful condition whether physical or psychological occurs, cortisol is released from the adrenal glands (Figure 10). The ability of the body to cope with this stress condition is dependent on several other factors such as the nutritional status, period of exposure to the stressor and lifestyle. However, the cortisol secreted together with other peptide hormones help to provide resistance to stress. Cortisol decreases the production of cytokines, mediators of inflammation and decreases effects of some inflammatory molecules on the target tissue. These various functions of cortisol in the stress response protects against damage from excessive inflammation and also enables the body to handle the stress condition by making more nutrients available to the body (150).
In a non-stressed condition, the amount of cortisol present in the blood undergoes a diurnal rhythm. The cortisol level rises to the highest levels in the early morning but drops in the evening and to its lowest a few hours after onset of sleep (153). However, in a chronic stress condition, there is accelerated conversion of PROG and a lack of diurnal rhythm, leading to an elevated plasma cortisol level. A breakdown of the negative feedback mechanism (section 3.2), exerted by the glucocorticoid, can cause several disorders which include memory loss, depression, Cushing’s disease, menstrual cycle abnormalities and anxiety and are characterized by elevated plasma cortisol levels (154).

3.4.2 Mineralocorticoids

The adrenal cortex secretes several different mineralocorticoids. Aldosterone is the principal endogenous mineralocorticoid and is responsible for a greater percentage of the total mineralocorticoid activity. Other endogenous hormone includes DOC, while fludrocortisone is a synthetic steroid with mineralocorticoid function (155). These mineralocorticoids play an important role in regulating the electrolyte concentration in the extracellular fluid, aldosterone having a greater potency (156). Acting on mineralocorticoid receptors (MR) on cells in the distal tubule of kidney nephron, aldosterone increases the expression of the epithelial sodium channel, facilitating the entrance of sodium ions. Aldosterone also functions in potassium and proton excretion and functions by increasing the expression of Na⁺/K⁺ ATPase molecules on the basolateral surface of these cells (157,158). The mineralocorticoid aldosterone acts on the CNS through the posterior pituitary, releasing vasopressin which helps to conserve water, while DOC found in the mammalian brain acts as a potent anti-convulsant (159). The absence of mineralocorticoid activities, leads to conditions such as high cellular fluid dehydration and low blood volume which can lead to circulatory shock and possibly death.

3.4.3 Androgens

The androgens include androstenedione, DHEA and its sulfo-conjugate (DHEAS) and testosterone (160). These hormones, which are also synthesized in the gonads, are responsible for the control of male sex trait and development as well as influencing the female sexual behavior. These hormones have varying potency and occur in different amounts in all vertebrates. Testosterone, which is the most potent androgen, is the major male sex hormone responsible for the normal growth and development of the male sex and reproductive organs.
This hormone is secreted primarily by the testes whereas the amount secreted by the adrenals is negligible and as such, not much of this hormone will be discussed in this section. In children, circulating DHEA and androstenedione are present at low concentrations. These two hormones are mostly inactive, having less than 20% of the potency of testosterone. However, since they serve as precursor for the active androgen, testosterones are regarded as adrenal androgens (161). The levels of the androgen DHEAS, however, increases considerably between the ages of seven and eight. This increase in concentration has been attributed to the role of DHEAS in increasing sexual development, sexual desire, strength, muscle mass, bone mass and growth (162). The level of DHEA and DHEAS reaches its peak between the ages of twenty to thirty during which time it is higher than any other steroid hormone. The circulatory level of these hormones starts to drop between the ages of forty to fifty years. The effect of the drop in the concentration level of these hormones leads to features such as loss of hair in the pubic areas, decreased sexual urge, loss of muscle and bone mass (163,164). Other functions of the adrenal androgens include regulation of gonadotropin feedback, brain function and maturation of the immune system. As mentioned earlier, the cytochrome P450 and the HSD enzymes are responsible for the biosynthesis of these adrenal hormones. The next section, will describe in detail the general biochemical properties of these enzymes.

3.5 Cytochrome P450 enzymes

The cytochrome P450 enzymes are members of a super family of heme-containing proteins found in bacteria, fungi, plants, and animals (165). The enzymes are present in a variety of organs and tissues – kidney, lungs, skin, adrenal gland, spleen, ovaries, testis, placenta and brain.

The first reported experimental study pertaining to P450 enzymes was in 1955 by Axelrod (166) and Brodie et al. (167). This study later identified an enzyme system in the endoplasmic reticulum of the liver, which oxidizes xenobiotic compounds. Two years later studies by Garfinkel (168) and Klingenberg (169) on P450 enzymes showed that the reduced enzyme, when complexed with carbon monoxide (CO), had an absorption maximum at 450 nm. The name P450 was thus derived from the characteristic feature of the enzyme absorbing light maximally at 450 nm. An electron spin resonance spectroscopy suggested that P450 was a low spin ferric hemeprotein (170) with a thiol residue as an axial heme ligand (171, 172, and 173).
The various reactions catalyzed by the cytochrome P450 enzymes include hydroxylation, epoxidation, deamination, desulfuration, dehalogenation, peroxxygenation and reduction. The reactions have a wide range of physiological functions including drug metabolism, detoxification of carcinogens and xenobiotics, as well as the metabolism of fatty acids, bile acids, vitamin D and steroids.

3.5.1 Steroidogenic Electron Transport

The cytochrome P450-dependent steroid hydroxylation reactions in the mitochondria and endoplasmic reticulum of adrenal cortex cells not only differ with respect to substrate specificity but there is a marked difference in the mechanism of the electron transport system. Thus they are broadly classified into four classes based on the electron transfer system they utilize.

The mitochondrial P450 enzymes, CYP11A1, CYP11B1 and CYP11B2, are regarded as Class I P450 enzymes and are characterized by a system which involves the transfer of reducing equivalents from NADPH to a flavoprotein, adrenodoxin reductase (AdxR) and subsequently to an iron sulphur protein, adrenodoxin (Adx) (Figure 12).

![Figure 12. Schematic representation of the mitochondrial electron transfer system. Flavin adenine dinucleotide (FAD); flavinmononucleotide (FMN); adrenodoxin reductase (Adx'); adrenodoxin (Adx); S, substrate (174).](image)

Adrenodoxin reductase is reduced by NADPH, which in turn reduces the adrenodoxin. Adrenodoxin forms a tightly associated biomolecular complex with adrenodoxin reductase.
The electron is transferred from adrenodoxin reductase to the oxidized adrenodoxin which, upon reduction, dissociates from adrenodoxin reductase. The reduced adrenodoxin subsequently associates with the substrate-bound cytochrome P450. The transfer of electrons thus involves sequential complex formations of adrenodoxin reductase and adrenodoxin with cytochrome P450, with the adrenodoxin molecule in the mitochondria electron transfer chain thus ‘shuttling’ the electrons between the adrenodoxin reductase and the P450 enzymes (174). The microsomal P450 enzymes are categorized as Class II P450 enzymes. The microsomal electron transfer system involves a single protein, cytochrome P450 oxidoreductase which contains two flavins. Electrons are transferred from NADPH to flavinadenine dinucleotide (FAD), subsequently to flavinmononucleotide (FMN) moiety and finally to cytochrome P450 and to the substrate (174). A schematic representation of the microsomal electron transfer system is shown below in Figure 13.

![Figure 13. A schematic representation of the microsomal electron transfer system. FAD, FMN and substrate (174).](image)

The class II enzymes are the most common in eukaryotes. The NADPH-P450 reductases and P450 enzymes are dissociated and bind to the outer-surface of the endoplasmic reticulum (ER) by amino-terminal hydrophobic bonds. These are typified by the liver microsomal enzymes in mammalian cells which are involved in both steroid metabolism and detoxification pathways.

The class III P450 enzymes such as P450 BM3 from *Bacillus megaterium* (175) require no electron donor. They are soluble and the enzyme and cofactors fused into one continuous polypeptide as shown in Figure 14.
Figure 14. A schematic representation of the class III P450 enzyme. Note the diflavin reductase is fused to the P450 (176).

The class III enzymes contain the same cofactors found in the class II enzymes but have a different primary structure (177). They are mostly involved in the conversion of peroxysgenated substrates which already contain oxygen and in the synthesis of prostaglandins in human (178).

The class IV enzymes, identified in *Rhodococcus sp*, receive electrons directly from NADH (176, 178). Enzymes in this class are also soluble and with cofactors fused to the heme domain as shown in Figure 15.

Figure 15. A schematic representation of the class IV P450 enzyme. Note an FMN-containing reductase with a ferrodoxin-like center linked to aP450 single polypeptide (176).

The class IV enzymes are involved in reactions such as reducing NO generated by denitrification to N₂O (178).
3.5.2 Physical properties of cytochrome P450 enzymes

The cytochrome P450 enzymes are made up of between 400-500 amino acids. These enzymes contain a single heme prosthetic group whose distal axial ligand is formed by a cystein residue (179). The protoporphyrin binds to a central iron atom which is a penta- or hexacoordinate. The presence of this complex contributes to the cytochrome P450 ability to cleave molecular oxygen (180). The mechanism at which substrates bind to cytochrome P450 enzymes and the subsequent formation and release of product, play an important role in the various functions of these enzymes. The bacterial cytochrome P450cam, which catalyses the hydroxylation of camphor, has been used as a prototype to understand the structure and function of the cytochrome P450 enzymes (181, 182).

Figure 16 Active site of substrate free P450cam. Oxygen atoms are shown in red, nitrogen in blue, sulphur in grey and iron in purple. Carbon atoms are shown in black while hydrogen is not shown (182).

3.5.3 Mechanism of P450 enzymes Catalyzed Reactions

The P450 enzymes catalyze reactions designed to promote the removal of lipophilic substances, including normal body constituents such as steroids and prostaglandins, together
with drugs and xenobiotics, from the body. They function as monooxygenases using the reduced NADPH as the electron donor for the reduction of molecular oxygen.

The general reaction is:

\[ RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \]

In the above reaction oxygen is activated by P450 and one oxygen atom is introduced into the substrate RH as a hydroxyl group, whereas the other atom of oxygen is reduced to H₂O. The electrons from NADPH are transferred to the substrate by two distinct electron transfer systems.

The replacement of an H-atom by a hydroxyl-group in the product decreases the lipophilic character of the substrate. Xenobiotics are made less lipophilic. The hydroxylated products of P450 enzymes are subsequently removed from the body either directly through the kidneys or by conjugation and subsequent secretion (183). Several studies have been done to investigate the P450 enzyme catalytic cycle. The mechanism of action of the hydroxylation reaction begins when a substrate binds to the active site, opposite the fifth ligand (Figure 17).

Figure 17. Schematic diagram showing the mechanism of action of P450 enzymes catalysing a hydroxylation reaction. RH is the substrate and ROH represents the hydroxylated product (183).
The redox potential is decreased which makes the transfer of an electron from its redox partner, NADH or NADPH favourable. The reaction proceeds with a change in the spin state of the heme iron at the active site, from a low-spin (LS) in which the five 3d electrons are maximally paired, to a high spin (HS) state in which the electrons are maximally unpaired. Binding of the substrate also brings about a conformational change in the enzyme which triggers an interaction with the redox component (184, 185).

The second stage in the cycle is the reduction of the iron by the electron transferred from NAD(P)H via the electron transfer chain to its ferrous form (Fe (II)). Molecular oxygen binds rapidly to the Fe\(^{2+}\) ion in the third step, forming Fe\(^{2+}\)-O\(_2\). There is evidence to suggest that this complex then undergoes a slow conversion to a more stable complex Fe\(^{3+}\)-O\(_2\)\(^-\) (186). The loss of the sixth coordinate allows oxygen to bind to the cytochrome P450 enzyme by heme ligation. The fourth stage is a second reduction step and has been determined to be the rate-determining step of the reaction (187). A comparison between the bond energies of the oxygen species formed, suggests that the Fe\(^{3+}\)O\(_2\)\(^-\) complex is the most favourable starting point for the next stage of the reaction to occur. The peroxo group, Fe\(^{3+}\)O\(_2\)\(^-\), is highly reactive and may associate with two protons in the environment of the active site to form hydrogen peroxide. The fifth step is a monooxygenase reaction in which the two oxygen atoms react with two protons from the surrounding solvent, cleaving the O-O bond, forming water and leaving an Fe-O\(^{3+}\) complex. In the sixth step, the Fe-ligated O atom is transferred to the substrate forming a hydroxylated form of the substrate. In step seven, there is the insertion of the hydroxyl product into the substrate.

### 3.5.4 Types of difference spectra identified in P450 enzymes

The P450 enzymes can be characterized using ultraviolet/visible spectrophotometry, identifying different intermediates in the catalytic cycle. In the oxidized state, in the absence of substrate, cytochrome P450 shows a Soret band at 419 nm which is associated with the LS state of the Fe (III). Nuclear magnetic resonance (NMR) and crystallographic results showed that a water molecule forms a sixth axial ligand of the Fe\(^{3+}\) in the substrate-free form, thus stabilizing the LS state of the ion (182, 188 189). The subsequent addition of substrate shifts the Soret band to 396 nm, attributed to cytochrome P450 being in the high spin form. Ligand binding to the P450 enzymes can be identified by observing the changes in the ultraviolet spectrum. There are three types of difference spectra which have been identified for Fe (III)
P450 — the type I, type II and the reverse type I (otherwise known as modified type II). These difference spectra depend on the type of ligand binding to the active site of the enzyme.

**Type I difference spectra**

The cytochrome P450 enzymes exist mostly in a low spin state with a ferric Soret absorbance maximum at approximately 418 nm. When ligands, such as oxygen containing substrates, bind to the molecule, they influence the iron spin-state equilibrium. These substrates change the spin equilibrium of the cytochrome P450 enzyme from a low to a high spin-state. This change is due to the shift of the water molecule out of the planar porphyrin towards the thiolate ligand. A type I substrate induced difference spectra is characterized with an absorbance maximum at 390 nm and minimum at 420 nm (Figure 18).

![Figure 18. Substrate-induced difference spectrum obtained by the addition of a substrate to cytochrome P450 (190).](image)

Most Cytochrome P450 enzymes substrates induce the high-spin state of the P450 enzymes and thus show a type I binding spectrum.

**Type II difference spectra**

The type II difference spectrum (Figure 19) is associated with the binding of inhibitors directly to the Fe$^{3+}$ displacing the water molecule as the sixth ligand (186, 191). Some of the inhibitors are N-containing compounds such as imidazole and amino-compounds. This
spectrum is identified by an increase in the absorption maximum between 425 nm and 435 nm and an absorption minimum between 390 nm and 405 nm (192). Unlike the type I difference spectra, type II difference spectra exhibits a shift to longer wavelengths indicating a change from a high spin state to a low spin state.

Figure 19. Type II difference spectra obtained by the addition of an inhibitor to cytochrome P450 (190).

Reverse type I (modified type II) difference spectra

Reverse type I difference spectra are obtained when high-spin state cytochrome P450 are bound to compounds which exhibit hydrophobic characteristics. The exact interaction of these ligands is not directly at the heme, but the ligand binds to a different site in the active pocket. The reverse type I spectrum can also be obtained when substrates dissociates from enzyme complex (193). It is also known as the modified type II and is characterized with an absorbance maximum between 409 nm and 445 nm and an absorbance minimum between 365 nm and 410 nm. The reverse type I closely resembles the type II binding spectrum.
3.5.5 Inhibitors of Cytochrome P450 enzymes

The cytochrome P450 enzymes are involved in several biological functions which includes the metabolism of endogenous steroids, dietary components and xenobiotic compounds. The catalytic activity of these enzymes can be reduced by the binding of inhibitors. Cytochrome P450 inhibitors include various chemicals, metabolic downstream products, drug molecules and natural occurring product found in diets or plant materials. Small molecules such as NO and CO can also bind to the enzyme and compete with oxygen in binding to the reduced heme iron (172). Other mechanism involved in the inhibition of cytochrome P450 enzymes by inhibitors includes heme ligation, heme adduct formation and formation of reactive intermediate.

The inhibitors can be categorized based on their mechanism as reversible or irreversible inhibitors. Generally, reversible inhibitors can be classified into three groups: competitive, mixed and non-competitive inhibition (194). However, the reversible inhibitors of the cytochrome P450 are mostly selective and inhibit competitively, binding to the enzyme with non-covalent interactions such as hydrogen and ionic bonds. These types of inhibitors are mostly similar in structure to the endogenous substrate. Unlike the reversible inhibitors, irreversible inhibitors such as CO, NO and cyanide inhibit the cytochrome P450 enzymes non-selectively, binding irreversibly to the heme iron and modifying the enzyme. The cytochrome P450 irreversible inhibition is usually obtained when inhibitors are activated by the enzyme, forming a reactive metabolite. This metabolite subsequently tightly bind to the enzyme active site, leading to a prolong inactivation. This process is known as mechanism based inhibition (195). A selective inhibitor for CYP21 known as 21, 21 dichloroprogesterone is an example of a mechanism based inhibitor. There is however a few examples in which an inhibitor of the cytochrome P450 enzyme might not necessarily be metabolized by the particular enzyme it inhibits. An example is quinidine which inhibits the CYP2D6 activity, but is metabolized by CYP3A (196).

The activities of the cytochrome P450 enzymes could lead to the activation of compounds to form carcinogenic/toxic species or to the accumulation of steroid hormones leading to side effects (section 3.4.1). Some of the important P450 isoenzymes enzymes such as CYP11B1 and CYP1A2 are linked to the onset of different types of cancer (197, 198). As such, the different inhibitors of these enzymes are used therapeutically for the treatment of several
diseases. Amphenone B was one of the earliest inhibitors studied and used clinically in the treatment of diseases associated with the adrenal cortex (199). This compound inhibits the catalytic activity of cytochrome P450 enzymes, diminishing the secretion of cortisol and aldosterone. Ascorbic acid and Cu$^{2+}$ are inhibitors of the 21-hydroxylase activity whereas studies done with compounds such as SU 8000 (3-(6-chloro-3-methyl-2-indenyl)) and SU10603 (chloro-3, 4-dihydro-2-(3-pyridyl)-1-(2H)-naphtalenone), shows that these compounds inhibit the lyase and hydroxylation reactions catalyzed by CYP17, thus blocking the synthesis of cortisol by the adrenal cortex. Metyrapone is a known as a selective inhibitor of CYP11B1. It is used as a therapeutic tool in Cushing’s syndrome a condition seen due to excess cortisol in the blood (200, 201).

Genistein, which has anti-tumorigenic properties, is a flavonoid commonly found in medicinal plants which also inhibits P450 enzymes (202). Other substances that can inhibit cytochrome P450 enzymes include carbon tetrachloride, flextime, ritonavir, barbiturates and naringenin which is found in grapefruit. These substances are non-selective inhibitors and readily bind irreversibly to the heme iron (203).

### 3.5.6 The catalytic role of cytochrome P450 enzymes in adrenal steroidogenesis

The cytochrome P450 enzymes involved in steroid hormone biosynthesis in the adrenal glands are specific in their choice of substrate. This section will describe the physiological functions of the various mitochondria and microsomal enzymes together with HSDs.

#### 3.5.6.1 CYP11A

This enzyme catalyzes the first reaction in the biosynthesis of steroids in the adrenals. This reaction involves the conversion of cholesterol to pregnenolone in a three sequential mono-oxygenations. First there are two hydroxylase reactions at positions 22 and 20 of cholesterol with the formation of 20α, 22-dihydroxycholesterol followed by a cleavage between C20 and C22 to form PREG and isocaproic aldehyde (204).
This reaction requires three molecules of oxygen, NADPH and the mitochondrial electron transfer system (Figure 20). The ability of CYP11A to catalyze the above reaction is dependent on the amount of available cholesterol in the inner mitochondria. The production of PREG is regulated by ACTH, which acts through the cAMP-mediated protein Kinase (PKA) pathway activating the steroidogenic acute regulatory protein (StAR) (205, 206 and 207). StAR mobilizes the transfer of cholesterol from the outer mitochondria to the inner mitochondria, hence, facilitating the catalytic production of PREG (208).

This enzyme is a product of a single gene and the cDNA was first isolated from bovine adrenal cortex mRNA (209). The structure of the cholesterol side-chain cleavage gene has been determined in human (209) and is expressed in all three zones, the zona fasciculata, the zona reticularis, and the zona glomerulosa in the adrenal cortex (210).

3.5.6 2 CYP17

This is a major enzyme that functions in key steps leading to the formation of the sex steroid hormones. CYP17 catalyzes two sequential reactions (Figure 21). The initial step is the $17\alpha$-
hydroxylation of C21 steroids, pregnenolone and progesterone. This step is crucial for the biosynthesis of cortisol while the second step is the cleavage of the intermediates 17-OH-PREG and 17-OH-PROG, at the C17-20 bonds to produce DHEA and androstenedione, respectively.

![Figure 21. Schematic representation of the hydroxylase and lyase activities of CYP17. (Arrow 1 represents the 17\(\alpha\)-hydroxylase activity and arrow 2 represents the C17,20-lyase activity).](image)

This reaction employs the microsomal electron transfer system and each reaction step utilizes one molecule of NADPH and one molecule of molecular O\(_2\) (211). Although CYP17 from various species all catalyzes the hydroxylation reaction, there are marked species-dependent differences in the use of either 17\(\alpha\)-hydroxypregnenolone or 17-OH-PROG as substrate for the lyase activity (174). In vitro studies performed on the CYP17 lyase activity, indicate that the human and bovine enzymes prefer 17-OH-PREG as substrate and forms DHEA as product whereas the rodent enzyme prefers 17-OH-PROG as the substrate forming androstenedione (212, 213).

3.5.6.3 CYP 21

This enzyme is responsible for the catalysis of the essential step in the synthesis of glucocorticoids and mineralocorticoids. In a similar reaction catalyzed by CYP17, CYP21
receives its electron from an NADPH-dependent Cytochrome P450 reductase (Figure 22), hence reducing molecular oxygen and hydroxylating the substrate (214).

Figure 22. Schematic representation of the 21-hydroxylation of progesterone and 17α-OH PROG.

CYP21 has a high degree of specificity and hydroxylates its substrates only at the C21 position. This enzyme is expressed in all three zones of the adrenal cortex and is also found in low levels in extra-adrenal tissues such as kidney, brain and liver (190).

3.5.6 4 CYP11B1 and CYP11B2

CYP11B1 and CYP11B2 are responsible for the essential and final reactions in the biosynthesis of cortisol and aldosterone respectively. CYP11B1 has the capacity for 11β-hydroxylation and is responsible for the catalytic conversion of 11-deoxycorticosterone and 11-deoxycorticisol to corticosterone and cortisol, respectively (Figure 23) (215).

Figure 23. Schematic representation of the enzymatic reaction catalyzed by CYP11B1 to yield corticosterone and cortisol.
These reactions require one molecule of oxygen and one molecule of NADPH. CYP11B1 can poorly catalyze the hydroxylation of C18 of 11-deoxycorticosterone or corticosterone to yield 18-hydroxycorticosterone, but does not catalyze the C18 oxidation to form aldosterone (216). However, CYP11B2 can catalyze both the 11β-hydroxylation and 18-hydroxylation. This is an essential step in the biosynthesis of aldosterone (Figure 24) (215).

\[ \begin{align*}
\text{11-Deoxycorticosterone} & \quad \text{O}_2, \text{NADPH} \quad \text{Corticosterone} \\
\text{18-Hydroxycorticosterone} & \quad \text{O}_2, \text{NADPH} \quad \text{Aldosterone}
\end{align*} \]

Figure 24. Schematic representation of the three step enzymatic reaction catalyzed by CYP11B2 to yield aldosterone.

This is a three step reaction with each step using one molecule of NADPH, one molecule of oxygen. The first reaction catalyzed is the 11β hydroxylation of 11-deoxycorticosterone, followed by the hydroxylation of C18 and an oxidation of the C18 hydroxyl group to yield the C18 aldehyde which leads to the formation of aldosterone. The major site of CYP11B1 expression is in the fasciculata and reticularis while CYP11B2 appears to be expressed predominantly in the glomerulosa cells (217).

3.5.6.5 The hydroxysteroid dehydrogenase

The hydroxysteroid dehydrogenase (HSD) enzymes, which include 3β-hydroxysteroid dehydrogenase (3β HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) play vital role in the biosynthesis of steroid hormones. These non-metallic enzymes are members of the same phylogenetic protein family known as the short-chain alcohol dehydrogenase reductase
superfamily (218). In the adrenals, they are involved in the catalysis of the last step in progesterone and androgen-androstenedione biosynthesis. This is a reduction and oxidation reaction that requires an NAD+/NADP+ as acceptors and the reduced forms as donors of reducing equivalents (Figure 25). Amongst this group of enzymes, 3β HSD contributes significantly to adrenal steroidogenesis. This enzyme catalyzes the conversion of the Δ5-3β-hydroxysteroids, PREG, 17-OH-PREG, and DHEA to the Δ4-3-ketosteroids, PROG, 17-OH-PROG, and A4 respectively.

![Chemical structures](image)

**Figure 25.** Schematic representation of reaction catalyzed by human 3βHSD I and II and mouse 3βHSD I and VI (219).

This conversion reaction is a two sequential reaction. First, there is the dehydrogenation of the 3β-equatorial hydroxysteroid, a step requiring the coenzyme NAD+ and yields a Δ5-3-ketosteroid intermediate and reduced NADH. In the next reaction, NADH activates the isomerization of the Δ5-3-ketosteroid to yield the Δ4-3-ketosteroid (219, 220). This reaction is catalyzed without the release of the intermediate or coenzyme (220). The P450 enzymes differs from the hydroxysteroid dehydrogenases in that they are the product of single genes, whereas there are several 3β HSD isoforms and several 17β-HSD isozymes, each a product of a distinct gene. 3β HSD/isomerases are membrane-bound enzymes and are distributed to both mitochondrial and microsomal membranes. Six different isoforms have been identified in mouse, and in human, two distinct isoforms of 3β HSD (3β HSD I and II) have been identified (221, 222 and 223).
3.6 Summary

The synthesis of steroid hormones in the adrenal gland is catalyzed by the cytochrome P450 enzymes. The production of these hormones is essential in order to maintain the normal physiological functions. These enzymes have a unique characteristic feature and are classified based on the electron transfer system they utilized. The catalytic activity of these enzymes varies and depends on substrate specificity, hormonal and some external regulatory factors.

The glucocorticoids, corticosterone and cortisol, are produced by the adrenals cortex after its activation by the HPA-axis. These hormones perform several physiological functions which in general, enables the body to handle the stress condition. However, in chronic stress condition, the negative feedback regulation of the HPA-axis is overridden resulting to increased levels of plasma glucocorticoids. Several disorders which include memory loss, depression, Cushing’s disease and anxiety have been attributed to this condition. Inhibition of the cytochrome P450 enzymes which are responsible for the biosynthesis of these steroid hormones has been employed therapeutically for the treatment of these disease conditions.

Several compounds are known to inhibit the catalytic activities of the cytochrome P450 enzyme. These compounds may inhibit the P450 enzymes by binding in the heme pocket of these enzymes or by other mechanism such as heme ligation, heme adduct formation and formation of reactive intermediate. The next chapter will discuss the inhibitory effect of *S. frutescens* extracts on the P450 enzymes.
CHAPTER FOUR

An investigation into the bioactivity of *S. frutescens*  
(Cancer bush)

4.1 Introduction

Alternative approaches to health care which make use of medicinal plants have been common in traditional cultures for centuries. Herbal remedies and plant-derived medicines have become increasingly popular as a means of medication, primarily due to increased concerns regarding the expense and toxicity of modern medicine. However, scientific evidence linking active compounds to pharmacological effects and modes of action have in most cases not been fully established.

*Sutherlandia frutescens* is one of the most well known indigenous medicinal plants used in traditional medicine (43). In South Africa, decoctions of the plant material are considered an effective and safe medicinal remedy used by traditional healers for the treatment of many ailments some of which include cancer, diabetes, anxiety and stress (44). *S. frutescens* has also been reported to stimulate the immune system, thus benefiting cancer and HIV patients (62, 224). The bioactive compounds in the leaves of *S. frutescens*, identified through chemical analyses, include canavanine, pinitol, triterpenoids and GABA. The biological activities of these compounds have identified *S. frutescens* as an important medicinal plant.

Many symptoms such as stress, depression and anxiety traditionally treated with *S. frutescens* can be associated with endocrine dysfunction. During the stress response, the biosynthesis of cortisol by adrenal gland is stimulated. Firstly, the hypothalamus is stimulated by a stressor to release CRH, which in turn binds to receptors on the corticotrophs in the anterior pituitary, resulting in the release of ACTH (150). ACTH stimulates the biosynthesis and release of cortisol by the adrenal glands. In chronic stress, the circadian rhythm is disturbed due to the sustained increased cortisol plasma levels and the negative feedback regulation of cortisol being over-ridden. Many disorders such as cardiovascular diseases, memory loss, depression, Cushing’s disease, menstrual cycle abnormalities and anxiety have been associated with persistent elevation of cortisol plasma concentrations.
The biosynthesis of cortisol and its precursors is catalyzed by the class I and II P450 enzymes in the adrenal gland. The initial step involves the conversion of cholesterol catalyzed by CYP11A to pregnenolone in the mitochondria. Pregnenolone moves to the cytosol where it is converted to 11OH-DOC and 11OH-deoxycorticisol by the class II P450 enzymes. These products re-enter the mitochondria where CYP11BI catalyzes the 11β-hydroxylation of these substrates, yielding corticosterone and cortisol, respectively.

CYP17 is a microsomal enzyme and is responsible for the catalysis of two mixed function oxidase reactions. The first reaction is the 17α-hydroxylation of C21 steroids, PREG and PROG, a crucial step for the biosynthesis of cortisol, followed by the cleavage of the intermediates 17OH-PREG and 17OH PROG, at the C17-20 bonds to produce DHEA and androstenedione, respectively. CYP 21 catalyzes the hydroxylation of C21 of PROG and 17OH PROG forming 11-DOC and 11-deoxycortisol. This is an essential step in the biosynthesis of glucocorticoids and mineralocorticoids (214).

Scientific research performed over the past few years to validate the effectiveness of S. frutescens as an anti-stress agent has shown promising results (7, 121). As discussed earlier in section 2.8, the results obtained from this study show that the use of S. frutescens led to a marked decrease in the corticosterone response in rats subjected to chronic intermittent immobilization stress. It is possible that bioactive compounds in S. frutescens may inhibit the adrenal P450 enzymes thus influencing cortisol biosynthesis.

This inhibitory effect may serve as a contributory factor leading to the anti-stress property of the plant material. Bioactive compounds can exhibit selective stimulation or inhibition of adrenal P450 enzymes and as such influence the outcome of adrenal steroid hormone biosynthesis. Some herbs have been shown to be either inducers or inhibitors of the P450 enzymes (224). The ability of several herbs, which includes grapefruit, Angelica dahurica and Gingko biloba, to inhibit P450 enzymes has been studied and their activities have been attributed to their bioactive compounds (225-228). Organic and hot aqueous decoctions of these herbs have also been shown to inhibit the P450 enzymes (229). Selective inhibitors of the P450 enzymes influencing the biosynthesis of glucocorticoids could thus be of therapeutic importance in the alleviation of various symptoms associated with elevated plasma glucocorticoids.
The bioactive compounds present in medicinal plants could interact with the P450 enzymes by preventing the binding of the natural substrates to the enzyme and subsequently influence product formation. Compounds may also bind to the enzyme while not interfering with the binding of the substrate and also affect the conversion of the bound. The bioactive constituents present in *S. frutescens* could thus inhibit the P450 enzymes in a competitive, non-competitive or mechanism-based, and as such used therapeutically for the treatment of diseases associated with elevated plasma glucocorticoids.

As discussed in chapter three, once a substrate is bound, the P450 enzymes exhibit type I, type II or reverse type I difference spectra. A type I spectrum with an absorbance maximum between the 385 nm and 395 nm and an absorption minimum at 420 nm is elicited by most P450 substrates, which upon binding displace the water molecule. The type II difference spectrum is characterized by a spectrum maximum between 425 nm and 435 nm and a trough at 390 nm and is elicited by substrates which bind directly to the Fe displacing the water molecule. Most type II ligands are known to be P450 enzyme inhibitors. The modified type II difference spectrum is characterized by an absorption increase at 420 nm and a decrease in absorbance at 390 nm. This is obtained when hydrophobic substrates bind to a site other than that of the substrate binding site and displace the water molecule by lipophylic interaction. The presence of an inhibitor which, together with the natural substrate, also binds to the active site can result in a reduction in the amplitude of the substrate induced difference spectrum, signifying an inhibitory effect on the P450 enzyme. It is also possible for an inhibitor to bind to the enzyme in the absence of substrate and elicit a spectrum.

Bioactivity assays of *S. frutescens* extracts were performed based on these unique spectroscopic features of the P450 enzymes. The spectrophotometric binding assays carried out in this study include (i) inhibitor-induced difference spectral assays which were performed to determine possible interactions of the extracts with the P450 enzymes and (ii) substrate binding assays performed in the presence of extracts.

Once the influence of *S. frutescens* extracts on the binding of natural substrates to the P450 enzymes is established, the influence on steroid metabolism will be assayed. In P450 enzyme catalyzed reactions, compounds which interact with the enzyme may decrease the catalytic activity of these enzymes. As discussed previously, these inhibitors can affect the catalytic
activity of the enzyme through different mechanisms. Most of the P450 inhibitors show a reversible inhibition, as they inhibit the enzyme competitively by binding to the active site of the enzyme. It is possible that inhibitors that do not resemble P450 substrates could inhibit the enzyme by binding to a site other than the active site, resulting in a non-competitive inhibition. Since S. frutescens have been used for the treatment of disorders attributed to elevated levels of glucocorticoids, this assay was performed to determine the effect of plant extracts on the conversion of the natural substrates. The ovine adrenal was used for this study since it is the only mammal that is related to human, which is easily accessible and can provide adequate amount of adrenals required for this study.

The aim of this research was to investigate the bioactivity of S. frutescens extracts on the adrenal P450 enzymes and on the metabolism of adrenal steroid hormones. Chloroform, methanol and aqueous extract of S. frutescens were prepared. The influence of S. frutescens was investigated on substrate binding to CYP17 and CYP11B1. In order to investigate the influence on adrenal steroid metabolism PROG, DOC and deoxycortisol conversion was subsequently assayed in ovine adrenal microsomes and mitochondria.

4.2 Materials and methods

4.2.1 Animal

Adrenal glands were collected from normal adult sheep at Paarl Abattoir in the Western Cape, South Africa. The surgically removed adrenals were immediately placed on ice and stored at −20 °C.

4.2.2 Plant material

Dried crushed S. frutescens subspecies microphylla1 leaves were collected from the Western Cape region of South Africa. S. frutescens tablets were purchased from Bioharmony (Phytonova) and Health Foods, South Africa.

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1 Kind gift from B-E van Wyk (Dept. Botany, University of Johannesburg, South Africa)
4.2.3 Reagents

Radioactive titrated steroid [3H] PROG was purchased from PerkinElmer Life Sciences (Boston, MA, USA). PROG, DOC, deoxycortisol, isocitrate, isocitrate dehydrogenase, cytochrome C, silica gel 60 F_{254} and PEG 8000 were purchased from Sigma Chemical Co. (St.Louis, MO, USA). NADH, NADPH and bovine serum albumin (BSA) were purchased from Roche Diagnostics (Germany). A bicinechonic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Scintillation fluid was purchased from Beckman Coulter Inc. (USA). Dialysis tube, Spectrapor No 1 was purchased from Spectrapor, Spectrum Medical Industries, Los Angeles, CA. All other chemicals were of reagent grade and purchased from either Sigma Chemical Co. or Merck Laboratory Supplies (RSA).

4.2.4 Equipment

The spectra were recorded using a Varian Cary 219 spectrophotometer. The radioactive titrated and non-titrated steroid metabolism products were analyzed using a Beckman Scintillation counter and an UN-SCAN-IT digitalizing software program.

4.2.5 Preparation of S. frutescens extracts

Chloroform, methanol and aqueous extracts

Chloroform and methanol S. frutescens extracts were prepared using a Soxhlet and a sequential extraction procedure was followed as previously described by Swart et al. (230). The soxhlet extractor, fitted with a double wall condenser, was connected to a round bottom flask. Dried S. frutescens plant material (18.9 g) was extracted with 250 ml chloroform at 40°C for 8 hours. The remaining plant material was subjected to further extraction with 250 ml methanol for 8 hours. The extracts were dried using a rotary evaporator after which the methanol extract (1.67 g) was re-dissolved with 31.45 ml of de-ionized water and the dried chloroform extract was re-dissolved in ethylene glycol such that both extracts had a final concentration of approximately 53 mg/ml.

Aqueous extracts were prepared by boiling 2.6 g of S. frutescens dried plant material in 275 ml de-ionized water in a beaker for 30 mins over a Bunsen burner. The extract was dried by
lyophilizing and the dried extract (0.64 g) re-dissolved in 12 ml of de-ionized water to a final concentration of approximately 53 mg/ml.

All the extracts preparations were centrifuged at 6000 × g for 5 min and filtered through a Whatman No1 filter paper and stored at -18 °C.

**Sutherlandia frutescens tablet extracts**

Tablets containing 300 mg of dried leaf powder were crushed and dissolved in 15 ml de-ionized water. The sample was incubated at 37 °C for 1 hr and centrifuged at 3000 × g for 5 min. The supernatant was lyophilized and 0.18 g of the dried extract was re-dissolved in de-ionized water to a final concentration of 53 mg/ml. The extract was stored at -18 °C till further use.

### 4.2.6 P450 enzyme preparation

Adrenals (25 g) were de-capsulated and the cortex homogenized in 75 ml 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM (EDTA) and 0.25 M sucrose using a Hamilton Beech blender followed by a Potter Elvehjem glass homogenizer. The homogenate was centrifuged at 1000 x g for 20 min. The supernatant fraction was subjected to further centrifugation at 12000 x g for 15 min. The supernatant was subsequently used for the preparation of microsomes while the pellet was retained for the mitochondrial preparation.

#### 4.2.6.1 Microsomal preparation

The microsomal preparation was carried out as previously described by Ming et al. and all procedures were carried out at 4°C using fresh sheep adrenal tissue (231). Polyethylene glycol (PEG) 8000, 50% (w/v) was added to the supernatant to a final concentration of 8.5%, stirred for 10 min at 4 °C and subsequently centrifuged at 13000 x g for 20 min. The supernatant was discarded, while the pellet was re-suspended and 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 mins at 4°C and centrifuged at 13000 x g for 20 min. This step was repeated in order to obtain a clear supernatant. The microsomal pellet was
re-suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.25 M sucrose and stored in 5 ml aliquots at -80 °C.

4.2.6.2 Mitochondrial preparation

The pellet containing the mitochondrial fraction was resuspended in 200 ml 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1% BSA and centrifuged at 12000 x g for 15 min. This step was repeated twice to obtain a clear supernatant. The mitochondrial pellet was subsequently lyophilized and the dried pellet homogenized in 24 ml dry acetone. The homogenate was centrifuged at 12000 x g for 15 min and the resulting pellet was lyophilized. The mitochondrial powder was stored at -18 °C.

4.2.7 Determination of cytochrome P450 concentration

The concentration of the P450 enzyme preparations was determined by the carbon dioxide method as described by Omura and Sato (190). The microsomal preparation was diluted in 0.1 M phosphate buffer (pH 7.4) containing 10% ethylene glycol saturated with CO and divided into two optically matched cuvettes. A baseline was recorded between 400-500 nm after which sodium dithionite (1-2 μg) was added to the sample cuvette and a spectrum recorded (232).

The mitochondrial powder (2 mg/ml) was first sonicated in 0.1 M phosphate buffer (pH 7.4) containing 10% ethylene glycol, in ice slurry for 5 min with one minute intervals. The spectral assay was performed as described above. The P450 enzyme concentrations of the preparations were calculated using a millimolar extinction coefficient (ε) of 91 using the equation below (232).

\[ \Delta A = \epsilon cl \]

Where \( \Delta A \) = change in absorbance

\( C \) = the concentration of the solution

\( l \) is the path of light through the solution (1 cm)

The protein concentration of the microsomal and mitochondrial preparation was determined using the Pierce BCA protein assay kit.
4.2.8 Preparation of Adrenodoxin and Adrenodoxin reductase (ADX/ADXR)

Since the adrenodoxin reductase (ADXR) and adrenodoxin (ADX) concentration in the mitochondrial preparation which was to be used to study the influence of *S. frutescens* on substrate conversion was inadequate, the protein had to be added to the reaction mixture when investigating the catalytic activity of the CYP11B1. The preparation of ADX/ADXR was carried out at 4°C using adrenal tissue as previously described by Omura *et al.* (15). Fresh sheep adrenals, 120 g, were de-capsulated and homogenized in 480 ml 0.25 M sucrose solution (pH 7.4) containing 10 mM EDTA. The homogenate was centrifuged at 1000 x g for 15 min. The supernatant was subjected to further centrifugation for 15 min at 9000 x g. The pellet obtained, was washed by re-suspension in 450 ml 0.25 M sucrose solution (pH 7.4) and centrifuged at 9000 x g for 15 min. The mitochondrial pellet was re-suspended in the smallest possible volume of 0.1M Tris-HCl buffer (pH 7.4) and sonicated. The sonicated fraction was centrifuged at 150 000 x g for 100 min, to yield a supernatant which contained ADXR and ADX. The activity of the ADXR/ADX-preparation was assayed as described below and as it was too dilute to use in the substrate conversion assays, the ADXR/ADX preparation was subsequently concentrated as described by Swart *et al.* (233). The assembled concentrator, shown in Figure 26, consists of four main components: an upper membrane support fitting, a lower membrane support fitting, the sample collecting chamber and a central spacing rod.

![Figure 26. Assembled perspex apparatus constructed for concentrating large volumes of dilute protein solution. (1) top membrane support fitting, (2) sample inlet, (3) O-ring, (4) central spacing rod, (5) O-ring, (6) clamps, (7) bottom membrane support fitting and (8) sample collecting chamber](image)
Dialysis tubing, Spectrapor No.1 (20.4 mm 6000-8000 Mr cut-off) used in the concentrator, was boiled in distilled water for 5 minutes and rinsed prior to use. Once the apparatus was assembled the dialysis tube was stretched and placed over the central spacing rod and the upper and lower membrane support fittings screwed onto either end of the rod. The ends of the dialysis tube were pulled over the upper and lower membrane support fittings so that 10 mm of the membrane covered both fittings. The O-rings were then placed over the tube covering the membrane support fittings and the dialysis tube sealed onto the supports by two circular clamps tightened by stainless steel screws. The sample collecting chamber was filled with 0.1M Tris-HCl buffer (pH 7.4) and gently connected to the lower membrane support fitting. The ADXR/ADX solution was subsequently loaded into the sealed dialysis tube through the solution inlet. With the vent was open the dialysis tube was filled to the lower edge of the upper membrane support fitting after which the vent was closed. The solution inlet was connected to the reservoir containing the dilute ADXR/ADX solution. The perspex apparatus was placed into a 1L beaker containing a 40% PEG solution (m/v in 0.1 M Tris-HCl buffer (pH 7.4) and stirred at 4°C as shown in Figure 27.

Figure 27. Experimental set-up for the concentration of ADX/ADXR solution.

Water which accumulated from the ADX/ADXR solution as a consequence of the osmotic pressure differential between the PEG solution and the dilute ADX/ADXR solution was removed. The concentration process continued until the ADX/ADXR solution in the sample solution reservoir was below the upper edge of the lower membrane support fittings. After completion of the concentrating process, the sample collecting chamber was detached from the lower membrane support fitting, the concentrated ADXR/ADX solution was collected and stored in 1 ml aliquots at -20°C.
4.2.9 Determination of Adrenodoxin/Adrenodoxin Reductase activity

The activity of the ADXR/ADX-preparation was assayed as previously described by Omura et al. (15). A reaction mixture containing ADXR/ADX (0.4 ml) and cytochrome c (20 mg/ml) in a final volume of 1 ml 0.1 M Tris–HCl (pH 7.4) was incubated at 25°C for 10 min. The mixture was subsequently divided into two optically matched cuvettes and a baseline recorded at 550 nm for 2 min. The reaction was initiated by the addition of NADPH (40 µl, 40 µM) to the sample cuvette. An equal volume of Tris-HCl buffer was added to the reference cuvette. The reduction of cytochrome c was monitored spectrophotometrically at 550 nm. The activity of the ADXR/ADX preparation was calculated from the amount of cytochrome c (nmol) reduced per min using a millimolar extinction co-efficient of 29.5. The assay was repeated in the presence of *S. frutescens* methanol extract (10 µl) to determine any possible influence in the reduction of cytochrome c. The protein concentration was determined using the Pierce BCA protein Assay kit.

4.2.10 Spectral binding assays

(i) Inhibitor-induced difference spectra:

The microsomal preparation was diluted to a final concentration of 0.7 µM cytochrome P450 with 0.1 M Phosphate buffer (pH 7.4) containing 10% ethylene glycol, 1.43 nmol P450/mg protein. The assay was carried out at room temperature in a final reaction volume of 1 ml using four optically matched cuvettes (2 reference and 2 sample cuvettes), in tandem. The influence of *S. frutescens* aqueous, methanol and chloroform extracts was assayed with the addition of 50 µl, to a final concentration of 2.65 mg/ml respectively. The extract was pipetted into a reference and a sample cuvette containing 0.2 M phosphate buffer (pH 7.4), while the remaining pair of cuvettes contained 1 ml of the enzyme preparation each. The baseline was recorded between 360 nm and 500 nm. The extract in the sample cuvette was replaced with 0.2 M phosphate buffer (pH 7.4) and 50 µl *S. frutescens* extract was added to the enzyme preparation in the sample cuvette while an equal volume of 0.1 M phosphate buffer (pH 7.4) was added to the enzyme preparation in the reference cuvette. The change in phosphate buffer concentration makes this assay more sensitive and less subject to interferences. Spectra were recorded over a period of 30 min until the spectra were completely developed.
(ii) Steroid-induced difference spectra

Mitochondrial and microsomal preparations were diluted to a final concentration of 0.7 µM P450 in 0.1 M phosphate buffer (pH 7.4) containing 10%ethylene glycol, 1.81 nmol P450/mg protein. Substrate binding was assayed at room temperature without and in the presence of aqueous, methanol and chloroform S. frutescens extracts, 20 µl, in a final concentration of 1.06 mg/ml. The assays were carried out in two optically matched cuvettes in a final reaction volume of 1ml and the baseline was recorded between 360 nm and 500 nm. Steroid substrates (32 µM) were added to the preparations in the sample cuvette - PROG to the microsomal preparation and DOC and deoxycortisol to the mitochondrial preparation. An equal volume of ethanol was added to the reference cuvette. The reaction mixture was mixed by inverting the cuvette and substrate-induced difference spectrum recorded between 360 nm and 500 nm.

A reduction in the amplitude of the spectrum - a decrease in the absorbance maximum at 383 nm and an increase in the absorbance minimum at 416 nm is indicative of inhibition of steroid binding to the cytochrome P450 enzymes and is calculated as follows:

\[
\% \text{ Inhibition} = 100 - \left( \frac{t}{c} \times 100 \right)
\]

Where:

\( t \) = absorbance at 390 nm minus absorbance at 420 nm in the presence of inhibitory component.

\( c \) = absorbance at 390 nm minus absorbance at 420 nm in the absence of inhibitory component.

(iii) Saturation substrate binding assays:

Saturation substrate binding assays were carried out using microsomal preparations diluted to a final concentration of 0.7 µM P450 in 0.1 M phosphate buffer (pH 7.4) containing 10% ethylene glycol, 1.43 nmol P450/mg protein. Binding assays were performed with varying PROG concentrations ranging from 2, 4, 8, 16, 32 to 64 µM. without and in the presence of S. frutescens extracts ranging from 2, 4, 6, 8 to 10 µl. In this assay, the S. frutescens extracts preparation used, were of a final concentrations as follows; aqueous extract 53 mg/ml,
methanol extract 37 mg/ml and chloroform extract 37 mg/ml respectively. The final concentrations of the aqueous extract was hence 0.106 mg, 0.212 mg, 0.318 mg, 0.424 mg and 0.53 mg and that of the methanol and chloroform extracts were 0.074 mg, 0.148 mg, 0.222 mg, 0.296 mg and 0.37 mg respectively. The assays were carried out at room temperature in two optically matched cuvettes. The amplitude of the type I difference spectrum was determined at each concentration without and in the presence of extracts. Hyperbolic saturation binding curves were generated by plotting the change in absorbance (390 nm-420 nm) against varying PROG concentrations. Double-reciprocal plots were generated to investigate the inhibitory effect of *S. frutescens* on the binding of PROG to the microsomal P450 enzymes.

4.2.11 Steroid conversion assays

(i) Steroid conversion assay in adrenal microsomes:

PROG metabolism was assayed in the microsomal preparation as described by Swart *et al.* (234). Radio-labelled titrated PROG (5000 cpm/50µl) and 10 µM PROG solutions were pipetted onto filter paper, dried under nitrogen and inserted into a test tube. PROG metabolism was assayed at 37°C with the addition of 50 mM Tris-HCl buffer (pH 7.4) containing 1% BSA and 50 mM NaCl, MgCl₂ (10 mM), isocitrate (2 mg/ml) and microsomal preparation (0.35 µM) to the dried steroids, in a total reaction volume of 1 ml. Assays were performed in the absence (control) of *S. frutescens* extracts and in presence of aqueous extract, 1.06 mg, chloroform extract, 0.74 mg and methanol extract, 0.74 mg respectively. After a 5 min pre-incubation period, an aliquot (50 µl) of the reaction mixture, which served as the initial time point, was taken. The reaction was subsequently initiated by the addition of NADPH (1 mM) and isocitrate dehydrogenase (0.2 U/ml). Aliquots (50 µl) of the reaction mixture were taken at 1, 4, 8 and 15 min. The samples were pipetted into glass screw cap test-tubes containing 5 ml dichloromethane and 450 µl distilled water, vortexed and centrifuged for 5 min. The aqueous layer was aspirated and the dichloromethane phase evaporated under a stream of nitrogen. The dried samples were subsequently re-dissolved in 100 µl dichloromethane for the extracted steroids subjected to TLC analysis.
Chromatography was performed on silica gel 60 F$_{254}$ plates. Steroid standards and samples (100 µl) were applied to the plates and separated in a solvent system (cyclohexane:ethyl acetate:ethanol (9:9:1)). The PROG, 17-OH-PROG, DOC and deoxycortisol metabolites were visualized using a UV light at 260 nm. The steroids were cut from the plates, placed in scintillation fluid (8 ml) and the radioactivity determined after 8 hrs using a Beckman Scintillation counter. Steroid metabolites were calculated as a percentage of the total of radioactivity detected for each steroid.

(ii) Steroid conversion assay in adrenal mitochondria

The conversion of the substrates, DOC and deoxycortisol (10 µM) was carried out in eppendorf tubes (1.5 ml) in an assay volume of 500 µl. The reaction mixture contained cytochrome P450 (0.7 µM), ADXR/ADX preparation (1320 U/ml), steroid substrates (5 mM), isocitrate (48 µM) and MgCl$_2$ (10 mM) in 0.05 M phosphate buffer (pH 7.4) The assays were performed in the absence (control) and in presence of aqueous extract, 1.06 mg, chloroform extract, 0.74 mg and methanol extract, 0.74 mg respectively.

Prior to the initiation of the reaction by the addition of NADPH (3 mM) and isocitrate dehydrogenase (1.875 U/ml) an aliquot of 50 µl was taken which served as the initial time point. Aliquots (50 µl) of the reaction mixture were taken at 1, 4, 8 and 15 min and steroid metabolites were extracted as described in the previous section. Samples were re-dissolved in 100 µl dichloromethane for TLC analysis.

The same TLC procedure as discussed above was used. Cortisol and corticosterone were visualized and the metabolites were analyzed using an UN-SCAN-IT digitalizing software program.
4.3 Results

Investigations into the bioactivity of *S. frutescens* were carried using assays based on the unique spectroscopic properties of the cytochrome P450 enzymes. The ability of plant extracts to bind and exhibit inhibitor-induced difference spectra was investigated to determine if *S. frutescens* bound directly to the enzymes. The influence of *S. frutescens* on the binding of natural substrates was subsequently investigated by determining the effect on substrate-induced difference spectrum. Finally, the conversion of natural substrates was assayed in microsomal and mitochondrial preparations containing the P450 enzymes. This assay was performed in order to determine the effect of the plant extracts on steroid biosynthesis.

4.3.1  Cytochrome P450 concentration in ovine adrenal mitochondria and microsomes

The cytochrome P450 content of the microsomal and mitochondrial enzyme preparations was determined by saturating the preparations with carbon monoxide prior to reduction by sodium dithionate (232). Sodium dithionate reduced carbon monoxide difference spectra of the enzyme preparations are shown in Figures 28 A and B. The spectra show the characteristic peak at 450 nm, indicating the presence of CYP 17 and CYP21 in the microsomal preparation and CYP11A, CYP11B1 and CYP11B2 in the mitochondrial preparation. Cytochrome P420, the inactive form of P450, which exhibits an absorbance maximum at 420 nm, was not detected.

Figure 28. Sodium dithionate reduced carbon monoxide difference spectra of ovine cytochrome P450. A: adrenocortical microsomal preparation, [Cytochrome P450] = 1.5 µM, 1.43 nmol P450/mg protein and B: adrenocortical mitochondrial preparation [Cytochrome P450] = 1.5409 µM, 1.81 nmol P450/mg protein.
The cytochrome P450 concentration, which was calculated using the millimolar extinction coefficient of 91 and the difference in the absorption between 500 nm and 450 nm, was 1.5 µM (1.43 nmol P450/mg protein) in the microsomal preparation and 1.5409 µM (1.81 nmol P450/mg protein) in the mitochondrial preparation.

4.3.2 Concentration of Adrenodoxin/Adrenodoxin Reductase

Since the concentration of ADX/ADXR was too dilute to be used in the steroid conversion assay in adrenal mitochondria, the protein was concentrated as discussed in section 4.2.8. The spectrophotometric assay was carried out at 550 nm to determine the activity of the concentrated ADX/ADXR as shown in Figure 29. Reduction of cytochrome c by ADX/ADXR is indicated by an increase in absorbance.

![Figure 29](image)

Figure 29. A: Cytochrome c reduction by ADX/ADXR electron transport system. B: Cytochrome c reduction in the presence of *S. frutescens* methanol extract. ADX/ADXR preparation, 400 µl, [protein] = 5.378 mg/ml, [NADPH] = 40 µM [cytochrome c] = 20 mg/ml. Methanol extract = 0.53 mg.

Cytochrome c was reduced by the ADX/ADXR electron system at a rate of 0.45 µmol per minute. In the presence of the methanol extract (Figure 29 B), no reduction of cytochrome c was observed. This is an indication that compounds in the extract could possibly interfere with the electron transport system and thus possibly inhibit the conversion of DOC and deoxycortisol to the respective metabolites.
4.3.3 Spectrophotometric binding assays

Inhibitor-induced difference spectra

Aqueous (2.65 mg), methanol (1.85 mg) and chloroform (1.85 mg) extracts were added to the ovine adrenal microsomes containing CYP17 and CYP21 to determine if these extracts could interact with the cytochrome P450 enzymes. Interaction of the *S. frutescens* extracts induced spectral changes as shown in Figure 30.

![Figure 30](image)

**Figure 30.** Difference spectra of microsomal P450 enzymes induced by *S. frutescens* extracts. A: aqueous extract, 2.65 mg; B: methanol extract, 2.65 mg; and C: chloroform extract, 2.65 mg [Cytochrome P450] = 0.7 µM.

The difference spectrum obtained with the aqueous extract showed an absorption maximum at 396 nm and although there was an interference of the aqueous extract pigmentation, the absorption minimum was recorded at 380 nm (Figure 30 A). Amongst the types of difference spectra identified in P450 enzymes, this difference spectrum closely resembles the reverse type I difference spectra (190). It is possible that the aqueous extracts could bind to the enzyme displacing the water molecule in the active site due to hydrophobic interaction. Upon the addition of the methanol extract, a difference spectrum with an absorption maximum
between 426 nm and 434 nm was obtained (Figure 30 B). The absorbance minimum could not be ascertained due to the pigmentation of the extract. However, this spectrum is comparable to type II difference spectra. The induced difference spectrum is possibly due to compounds in the methanol extract binding directly to the ferric ion and displacing the water molecule. The spectrum induced by the chloroform extract (Figure 30 C) exhibits an absorption minimum at 420 nm which is indicative of a type I spectrum. The absorbance maximum normally observed between 390 nm and 380 nm cannot be easily ascertained, possibly due to pigments in the chloroform extract interfering with the absorbance at this wavelength.

**Inhibition of steroid-induced difference spectra**

Once the interaction of the different *S. frutescens* extracts with the P450 enzymes had been established, the inhibitory effect of the *S. frutescens* extracts on the binding of natural substrates to the enzyme was determined. The binding of PROG (32 µM) to microsomal P450 enzymes induced a typical type I difference spectra with an absorbance maximum at 387 nm and minimum at 419 nm as shown in Figure 31 A.

**Figure 31.** A: Inhibition of PROG-induced type I difference spectra in adrenal microsomes [cytochrome P450] = 0.7 µM, [PROG] = 32 µM ( ), aqueous extract, 1.06 mg ( ), methanol extract, 1.06 mg ( ) and chloroform extract, 1.06 mg ( ). B: Percentage inhibition of PROG binding to microsomal P450 enzyme by *S. frutescens* extracts. Results are the averages of three independent experiments.

Inhibition of PROG binding by the different *S. frutescens* extracts is shown by the reduction in the amplitude of the peaks. The chloroform extract showed an inhibition of 52% which was
significantly (P < 0.001) higher than the inhibition by the methanol (22%) and aqueous extracts (18%) (Figure 31 B). The inhibition of PREG binding to the microsomal P450 enzymes was also carried out (data not shown), however the different *S. frutescens* extracts could not affect PREG binding to the enzyme.

The ovine adrenal mitochondrial cytochrome P450 substrate-induced difference spectra are shown in Figure 32.

Figure 32. Inhibition of steroid-induced type I difference spectra by *S. frutescens* extracts in adrenal mitochondrial preparation. A: Inhibition of DOC-induced difference spectra and B: Percentage inhibition of DOC binding to CYP11B1, [DOC], 32 µM (—); C: Inhibition of deoxycortisol-induced difference spectra and D: Percentage inhibition of deoxycortisol binding to CYP11B1 [deoxycortisol], 32 µM (—). [Cytochrome P450] = 0.7 µM, aqueous extract, 1.06 mg (—), methanol extract, 1.06 mg (—) and chloroform extract, 1.06 mg (—). Results are representative of three independent experiments.

Both DOC and deoxycortisol induced a type 1 difference spectrum when the substrates bound to CYP11B1 in the absence of *S. frutescens* extracts (Figure 32A and C). Inhibition of steroid
binding to CYP11B1 in the mitochondrial preparation was observed by the reduction of the peak amplitude in the presence of the extracts. The chloroform extract inhibited the binding of DOC by 52%, aqueous extract inhibited DOC binding by 8.5% while inhibition by methanol extract was at 10.7%. The inhibition of DOC binding by the *S. frutescens* chloroform extract was significantly higher (P < 0.001) than the inhibition by the *S. frutescens* aqueous and methanol extracts.

The inhibition of deoxycortisol binding to CYP11B1 by the chloroform extract (42.3%) and methanol extract (37.9%) was significantly (P < 0.001) different to the inhibition exhibited by the aqueous extract. Interestingly inhibition of deoxycortisol binding to CYP11B1 by the *S. frutescens* methanol extract was significantly greater than that of DOC binding to CYP11B1 (P < 0.001).

The inhibition of steroid binding to ovine adrenal mitochondrial and microsomal P450 enzymes in the presence of extracts prepared from processed *S. frutescens* in the form of commercially available tablets was also investigated. DOC, deoxycortisol and PROG binding to ovine adrenal mitochondrial and microsomal P450 enzymes was assayed to determine whether manufacturing processes could affect the bioactivity of the plant material. The type I induced spectra exhibited by the binding of the steroid substrates to the mitochondrial and microsomal P450 enzymes in the presence of these extracts is shown in Figure 33 A, B and C.
Figure 33. Inhibition of substrate-induced type I difference spectra in ovine adrenal mitochondria [Cytochrome P450] =0.7 µM, A: [DOC] = (•••••) 32 µM. B: [deoxycortisol] = (•••••) 32 µM. C: [PROG] = (•••••) 32 µM. S. frutescens tablets extract, 1.06 mg (■■■). D: Percentage inhibition of DOC, deoxycortisol and PROG binding to mitochondrial and microsomal P450 enzymes. Results are representative of three independent experiments.

Varying degrees of inhibition on substrate binding was observed in the presence of the S. frutescens tablet extract. Hence it could be deduced that manufacturing processes may have negligible influence on the bioactivity of the S. frutescens tablet extract. The extract inhibited the binding of DOC by 21.97%, deoxycortisol by 19.81% and PROG by 12.97%. Statistical analysis however shows that the inhibitory effect of this extract on the binding of these steroids is not significantly different (P > 0.05).

**Saturation binding assay**

Since the S. frutescens extracts were shown to inhibit the binding of endogenous steroid substrates to the cytochrome P450 enzymes, the inhibitory effects were further investigated in order to establish the type of enzyme inhibition. The inhibition of PROG binding to the microsomal enzymes was therefore assayed in the presence of various substrate and extract concentrations.
Upon addition of increasing PROG concentrations the amplitude of the induced type I difference spectra increased. Hyperbolic saturation binding curves were generated by plotting the change in absorbance (390 nm-420 nm) against varying concentration of substrate using the equation: \( \Delta \text{Abs} = \frac{B_{\text{max}}[S]}{K_{s}} + [S] \).

In the above equation, the change in absorbance (\( \Delta \text{Abs}, 390 \text{ nm}-420 \text{ nm} \)) is the amplitude of the binding spectrum, \( B_{\text{max}} \) is the maximum substrate binding capacity of the enzyme while the \( K_{s} \) is the substrate dissociation constant (Figure 34 A, D and G). A decrease in the maximum substrate binding capacity was observed upon addition of increasing concentrations of \( S. frutescens \) extracts. The substrate binding data was subsequently plotted on Double-reciprocal plots (using equation \( y = mx + c \)) were generated (Figure 34 B, E and H) to visualize the inhibition data.
Figure 34. Hyperbolic saturation binding curves of the inhibitory effect of \textit{S. frutescens} extracts on PROG binding to microsomal P450 enzymes: A: aqueous extracts; D: methanol extracts; and G chloroform extract. Double reciprocal plots of the inhibitory effect of \textit{S. frutescens} extracts on PROG binding: B: aqueous extracts E: methanol extracts; H: chloroform extracts. Secondary plot of the slope of $1/\Delta$Abs against inhibitor concentration: C: aqueous extracts; F: methanol extracts; I: chloroform extracts. [Cytochrome P450] = 0.7 µM; [PROG], (2 µM – 64 µM); aqueous extracts, 0.106 mg - 0.53 mg; methanol extracts, 0.074 mg - 0.37 mg; and chloroform extracts 0.074 mg - 0.37 mg. Results are representative of two independent experiments.

In the presence of increasing concentrations of \textit{S. frutescens} extracts, a concentration dependent inhibition of PROG binding to the cytochrome P450 enzyme was observed. This inhibition was characterized by a concentration-dependent increase in the substrate dissociation and a corresponding decrease in the apparent affinity of the cytochrome P450 enzymes for the substrate. The results obtained with the binding assays which were conducted is only a reflection of binding inhibition and is not an indication of whether the catalytic
activity is influenced, although it could be assumed that if the binding of the substrate is affected the activity would also be affected – substrate conversion may be inhibited or the rate of substrate conversion may be lower. The mode of inhibition is consistent with that of mixed inhibition. The associated Ki’ values were obtained from secondary plot of \(1/ (\Delta \text{Abs, 390 nm-420 nm})\) against the concentration of \(S. \text{frutescens}\) extracts. The Ki’ values for the aqueous, methanol and chloroform extracts (Figure 34 C, F and I) were 0.9, 0.12 and -0.002 respectively and from these values it can be deduced that the chloroform extract exhibits greater interaction with the enzyme substrate complex. The corresponding Ki values were obtained by plotting the \(K_m/B_{max}\) values against the inhibitor concentration (I) as shown in Figure 35 A, B and C.

![Figure 35. Secondary plot of the \(K_m/B_{max}\) values against inhibitor concentration \([I]\): A: aqueous extracts; B: methanol extracts; C: chloroform extracts. \([\text{Cytochrome P450}]=0.7 \mu \text{M}; [\text{PROG}], (2 \mu \text{M} – 64 \mu \text{M}); \text{aqueous extracts, 0.106 mg - 0.53 mg; methanol extracts, 0.074 mg - 0.37 mg; and chloroform extracts 0.074 mg - 0.37 mg. Results are representative of two independent experiments.}]

The Ki values for the \(S. \text{frutescens}\) aqueous, methanol and chloroform extracts (Figure 35 A, B and C) were 0.2821, 0.0021 and 0.0814 respectively. This data indicates that the \(S. \text{frutescens}\) methanol extract which had the lowest Ki value, shows a higher inhibition of PROG binding to the P450 enzyme.

### 4.3.4 PROG metabolism in ovine adrenal microsomes

It is possible that compounds in the extracts can bind to the enzymes and thus prevent substrates from binding or compounds can bind to the enzyme substrate complex and not affect substrate binding, as is possibly the case observed with PREG (7). Although spectrophotometric assays of compounds with the P450 enzymes can be carried out to
determine the effect on substrate binding, the influence on the conversion of substrates will still need to be established.

Once the inhibition of PROG binding to ovine adrenal microsomal enzymes, CYP17 and CYP21, by the *S. frutescens* extracts had been established, further investigation into the influence of these extracts on the catalysis of steroid substrates by these enzymes was undertaken. CYP17 catalyzes the 17α-hydroxylation of PROG to yield 17-OH-PROG while CYP21 catalyzes the hydroxylation of PROG and the product of CYP17 to produce 11-deoxycorticosterone and 11-deoxycortisol respectively. Inhibition of the activity of CYP17 will lead to the production of mineralocorticoids and glucocorticoids while inhibition of the catalytic activity of CYP21 will result in the inhibition of these steroid intermediates and production of androgens. On the addition of PROG to the microsomal preparation, as shown in Figure 36, both CYP17 and CYP21 catalyzed the conversion of the steroid substrate.

![Figure 36. Metabolism of PROG (10 µM) in ovine adrenal microsomes after initiation with NADPH (1 mM). Cytochrome [P450], 0.7 µM.](image)

After 4 minutes, 35% of the PROG had been converted to 17-OH-PROG (18%), DOC (9%) and deoxycortisol (8%). After 15 min, only 6.37% PROG remained, with most of the substrate being converted to DOC (54.4%) and the 17-OH-PROG intermediate which had
formed was converted to deoxycortisol (33.4%). The inhibition of the catalytic activity of CYP17 and CYP21 is shown in Figure 37.

Figure 37. Metabolism of PROG (10 µM) in ovine adrenal microsomes after initiation with NADPH (1 mM) in the presence of A: chloroform extract; B: methanol extract; C: aqueous extract. Percentage inhibition of steroids in the presence of D: chloroform extract; E: methanol extract; F: aqueous extract. Cytochrome P450, 0.7 µM. chloroform extract, 0.74 mg; methanol extract, 0.74 mg; and aqueous extract, 1.06 mg. Results are representative of three independent experiments.
In the presence of *S. frutescens* extracts, as shown in Figure 37 A, B and C, the formation of DOC and deoxycortisol is clearly inhibited as negligible amounts of the steroid products are formed. In the presence of aqueous extract, which had a higher concentration (final concentration 1.06 mg extract/ml) (Figure 37C), 55% of the PROG was converted to 17-OH-PROG (25.5%), DOC (9.1%) and deoxycortisol (19.3%).

On completion of the reaction, the data shows that the inhibition of the catalytic activity of CYP21 towards PROG in the production of DOC by the different *S. frutescens* extracts, was greater than the inhibition of the catalytic activity of CYP17 in the production of 17-OH-PROG (P < 0.001) (Figure 37 D, E and F). Furthermore, the production of deoxycortisol was also markedly inhibited by these extracts more than the inhibition of 17-OH-PROG (P < 0.001), indicating that these extracts are more potent inhibitors of CYP21 when compared to their inhibitory effects on CYP17.

**Inhibition of PROG metabolism by *S. frutescens* tablet extracts**

Since the *S. frutescens* tablet extracts could inhibit the binding of steroids to the cytochrome P450 enzymes as previously shown, the conversion of PROG in ovine adrenal microsomes was assayed in the presence of the extract. In the presence of *S. frutescens* tablet extract, the metabolism of PROG was inhibited as illustrated in Figure 38 B.

![Figure 38. Metabolism of PROG (10 µM) in ovine adrenal microsomes after initiation with NADPH (1mM). Cytochrome P450, 0.7 µM. A: *S. frutescens* tablet extract, 6.6 mg and B: percentage inhibition of PROG metabolism. Results are representative of three independent experiments](image)

Although a higher concentration of the *S. frutescens* tablet extract was used as compared to the *S. frutescens* aqueous, methanol and chloroform extracts, a similar inhibitory effect on PROG metabolism is shown. Statistical analysis indicates that inhibition of the catalytic activity of CYP21 towards the production of both DOC and deoxycortisol by the *S. frutescens* tablet extract was similar to the other extracts.
tablet extract was significantly higher than the inhibition of CYP17 towards the production of 17-OH-PROG (P < 0.001).

4.3.5 Steroid conversion assay in adrenal mitochondria

DOC and deoxycortisol are the products of CYP17 and CYP21 catalyzed hydroxylase reactions, respectively. These intermediates move into the mitochondria and undergo a hydroxylation reaction catalyzed by CYP11B1 to form corticosterone and cortisol respectively. Since the *S. frutescens* extracts interacted with these enzymes and inhibited the binding of these substrates, the influence on the catalytic activity of CYP11B1 was investigated as shown in Figure 39.

Figure 39 Metabolism of steroid substrates by ovine adrenal mitochondrial enzyme after initiation with NADPH (1 mM) A: DOC metabolism in the absence of extracts [DOC] = ( ) 10 µM. C: deoxycortisol metabolism in the absence of extracts [deoxycortisol] = ( ) 10 µM; B: percentage inhibition of DOC in the presence of chloroform extract, methanol extract and aqueous extract and D: percentage inhibition of deoxycortisol in the presence of chloroform extract, methanol extract and aqueous extract. Cytochrome [P450], 0.7 µM. Chloroform extract, 0.74 mg; methanol extract, 0.74 mg; and aqueous extract, 1.06 mg. Results are representative of three independent experiments.
After the incubation of the enzyme with the steroid substrates in the absence and presence of *S. frutescens* extracts, the amount of steroid metabolized were analyzed using an UN-SCAN-IT digitalizing software program. In the absence of *S. frutescens* extracts, 60.7% DOC and 30.24% deoxycortisol had been metabolised to corticosterone and cortisol respectively. In the presence of *S. frutescens* chloroform and methanol extracts, both the inhibition of DOC and deoxycortisol metabolism was significant (p < 0.001) with negligible amounts of products being formed. However in the presence of the aqueous extract, there was a lesser degree of inhibition as 45.6% DOC was converted to corticosterone and 16.14% deoxycortisol was converted to cortisol as shown in Figure 39 B and D.
4.4 Discussion

The synthesis and release of adrenal glucocorticoids is most common in human and other mammals. Psychological or acute physiological stress activates the HPA which subsequently results in an increase in plasma cortisol level. Several diseases have been attributed to this condition. The increase of cortisol secretion is regulated by ACTH, which also increases the synthesis of the adrenal P450 enzymes. These cytochrome P450 enzymes are responsible for the catalytic reactions in steroid hormone biosynthesis and as such inhibition of these enzymes is of therapeutic importance for the treatment of these diseases.

*Sutherlandia frutescens* is one of several medicinal plants used for healthcare practices and in order to investigate the bioactivity of the plant material, assays were carried out to determine if the plant extracts could in the absence of natural substrate, bind directly to the P450 enzymes. Each of the different *S. frutescens* extracts was able to induce a particular type of inhibitor-induced difference spectrum on interaction with the enzyme. The methanol extract induced a type II difference spectrum. The binding of this extract or compounds in this extract, results in a shift to a longer wavelength, with the ferric iron changing from a high-spin state to a low-spin state, and at this point, shifting into the plane of the heme which favors the low-spin state. It is likely that the methanol extracts inhibits the cytochrome P450 enzymes by binding directly to the Fe$^{3+}$ and displaces the water molecule. When the *S. frutescens* chloroform extract was assayed in the microsomal preparation, a type I difference spectrum was obtained. It is possible that hydrophobic compounds present in the chloroform extract could cause a shift of the Fe$^{3+}$ spin equilibrium from a low spin state to a high spin state. In this case, the water molecule is displaced from the sixth position and the iron shifts out of the planar protoporphyrin towards the fifth ligand. In contrast the *S. frutescens* aqueous extract when assayed, showed a reverse type I difference spectrum. The binding of this extract tends to favor a spin equilibrium shift of the ferric iron to its low-spin state. It is possible that the hydrophilic compounds in the aqueous extract do not bind directly to the heme, but rather displace the water molecule and bind to a different site in the heme pocket.

Since the interaction of the extracts had been established previously, further investigations on the effect of the extracts on the binding of natural steroids to the cytochrome P450 enzymes were carried out. The three *S. frutescens* extracts prepared in this study were able to inhibit the binding of PROG to the P450 enzymes in the microsomal preparation. Since the
interaction of these extracts with the cytochrome P450 enzymes leads to the inhibition of PROG binding, it could be possible that compounds in the S. frutescens extracts, once bound to the enzyme, brings about a conformational change of the enzyme and hence hinders the binding of the endogenous steroid. It is also possible that these extracts could bind to a site different from the active site and thus prevent the binding of the endogenous steroid substrate. Both DOC and deoxycortisol induced a type I difference spectrum when the substrates bound to CYP11B1 in the absence of S. frutescens extracts. Similarly, the S. frutescens extracts were able to inhibit the binding of these substrates to the cytochrome P450 mitochondrial enzyme. However, in a similar study by Prevoo et al. (7), when the inhibition of PREG binding was assayed in the microsomal P450 enzymes, there was no significant inhibition by the S. frutescens extracts, indicating that it is possible that these extracts can bind to the enzyme substrate complex but may not affect the substrate from binding to the P450 enzyme. Inhibition of steroid substrate binding to the microsomal and mitochondrial P450 enzymes was significantly greater by the chloroform and methanol extracts (P <0.001) compared to the aqueous extract. It is possible that the hydrophobic molecules present in these S. frutescens extracts prevent the various steroids from binding to the P450 enzymes.

During the commercial manufacturing process, several factors such as growth, time and method of collection, drying, packing and storage can affect the efficacy and the therapeutic value of medicinal plants. In order to determine if the bioactivity of S. frutescens is affected by these factors, S. frutescens tablets extracts were assayed with microsomal and mitochondrial preparations. The recommended therapeutic dose of S. frutescens for an adult is 9 mg/kg/day, however, in this study a lower concentration of 1.06 mg was used. The data obtained from the investigations into the influence of the extracts on substrate binding indicates that the S. frutescens tablet extract was able to inhibit the binding of PROG, DOC and deoxycortisol to the microsomal and mitochondrial cytochrome P450 enzymes. Although the inhibition of DOC binding to CYP11B1 by the extract seemed to be the highest, statistical analysis indicates that the difference in the level of steroid inhibition by this extract was not significant. This inhibitory effect shown by the extract indicates that the manufacturing process might have a negligible effect on the bioactivity of S. frutescens.

The addition of the native steroid substrate induced typical type I substrate induced difference spectra in both the mitochondrial and microsomal adrenal preparations. In the presence of the S. frutescens tablet extracts, a decrease in the amplitude of the peaks was observed indicating
inhibition of steroid binding. Thus, in an effort to understand the mode of enzyme inhibition of the P450 enzymes by the various *S. frutescens* extracts, a saturation substrate binding assay was carried out. Enzyme inhibitors are an important class of pharmacological agents. These inhibitors act in variety of ways. These mechanisms depend on the competition between the substrate and inhibitor for the binding site, the formation of an enzyme/inhibitor complex or an enzyme/substrate/inhibitor complex and the manner in which the inhibitor affects the enzyme. Inhibitors of cytochrome P450 enzymes generally inhibit in a reversible manner. The double reciprocal plot obtained indicates that the various *S. frutescens* extracts exhibit mixed inhibition, which is a combination of different types of reversible enzyme inhibition; competitive, non-competitive and uncompetitive inhibition. These *S. frutescens* extracts can bind to either the free enzyme or the enzyme-substrate complex. This form of inhibition was characterized by a concentration-dependent increase in the substrate dissociation and a corresponding decrease in the apparent affinity of the cytochrome P450 enzymes for the substrate. It is also possible that these extracts or compounds in these extracts bind to a site different to the site to which the native substrate binds in the active pocket. From the Ki' values obtained, the *S. frutescens* chloroform extract which has the lowest value has the highest interaction with the enzyme substrate complex, while the *S. frutescens* methanol extract which has the lowest Ki value interacts more with the enzyme and is the most potent of all the three *S. frutescens* extracts.

Since the interaction of the *S. frutescens* extracts resulted in an inhibition in steroid binding to the P450 enzymes, steroid metabolism, which is a measure of biological activity, was subsequently investigated to determine the biological effect of the *S. frutescens* extracts towards the catalytic activity of the cytochrome P450 enzymes. When PROG metabolism was assayed in the absence of *S. frutescens* extracts, CYP17 catalyzed the hydroxylation of PROG to yield 17-OH-PROG while CYP21 catalyzed the hydroxylation of PROG and 17-OH-PROG to yield DOC and deoxycortisol respectively (235). The rate of PROG conversion by CYP21 was observed to be higher than that of the CYP17 and most of the PROG as well as the 17-OH-PROG produced, was converted to DOC and deoxycortisol. Interestingly, upon the addition of the different *S. frutescens* extracts, the inhibition of the catalytic activity of CYP21 was significantly higher (P < 0.001) than the inhibition of the catalytic activity of CYP17. Statistical analysis further showed that the inhibition of the DOC and deoxycortisol production was significantly higher (p<0.001) by the chloroform extract.
The *S. frutescens* tablet extract was also able to inhibit both the CYP17 and CYP21 hydroxylase activities of the enzymes. Interestingly just like the aqueous and organic *S. frutescens* extracts, inhibition of the catalytic activity of CYP21 towards the production of both DOC and deoxycortisol was significantly higher (P < 0.001) than the inhibition of CYP17 towards the production of 17-OH-PROG. This data suggests that the bioactive components in the plant material are not influenced during manufacturing process.

The CYP11B1 catalyzes the catalytic conversion of DOC and deoxycortisol to corticosterone and cortisol respectively. The reaction requires one molecule of oxygen and one molecule of NADPH. This enzyme uses the mitochondrial electron transfer system which consists of ADXR, a FAD-containing reductase, and ADX, a soluble, low molecular weight iron sulphur ferrodoxin-type electron transfer protein. In this study, a concentrated ADXR/ADX solution was prepared and the activity characterized by its ability to reduce cytochrome c. This assay was also carried out in the presence of *S. frutescens* methanol extract in order to determine any possible effect of the plant material towards the reduction of cytochrome c by ADX/ADXR. It is possible that since oxidized cytochrome c does not absorb at 550 nm, the inability of an absorbance to be recorded was due to interference of the methanol extract with the electron transport system.

Once the activity of the ADX/ADXR solution had been established, DOC and deoxycortisol metabolism was assayed in the presence of *S. frutescens* extracts. DOC and deoxycortisol metabolism were significantly inhibited (p < 0.001) by the methanol and chloroform extracts with negligible amounts of products being formed. However, the inhibition recorded in the presence of aqueous extract was significantly lower than the inhibitory effect of chloroform and methanol extracts. It is possible that the binding of these inhibitors compromises the catalytic activity of the P450 enzymes and slows down the rate of product formation. This data suggests that *S. frutescens* extracts could be potent CYP11B1 inhibitors that block the biosynthesis of corticosterone and cortisol.

In summary, from the data presented in this study it is evident that *S. frutescens* extracts were able to interact with the cytochrome P450 enzymes and could inhibit steroid binding to ovine adrenal cytochrome P450 enzymes. The data provided in this study also provides an insight to the mode of inhibition of the *S. frutescens* extracts on the binding of endogenous steroid to the P450 enzymes. The *S. frutescens* extracts were also able to inhibit the various catalytic
activities of the P450 enzymes involved in the biosynthesis of cortisol, indicating the plant may ultimately affect the biosynthesis of glucocorticoids and influence the elevated blood cortisol levels associated with chronic stress.
CHAPTER FIVE

Conclusion

*Sutherlandia frutescens* is an indigenous plant of South Africa and is commonly known as Cancer bush or Unwene. The plant material which was first used by the Khoi and Nama people for the washing of wounds and stomach problems, have also been shown to be useful for the treatment of cancer, diabetes, tuberculosis, heartburn, stress and stress related diseases. *S. frutescens* has enjoyed a long history of use due to the aforementioned medicinal applications as well as the safety and efficacy of the plant material and the standardization of commercially available *S. frutescens*. The various bioactive compounds identified in *S. frutescens* are known to exhibit several biological activities. Several *in vitro* and *in vivo* studies have been carried out, demonstrating a range of pharmacological activities supporting the wide use of the plant material. These studies focused on areas such as the anti-cancer, anti-diabetic, anti-inflammatory, anti-convulsant, anti-HIV and anti-bacterial activities of the plant material. Experiments performed by Smith *et al.* (121) and Prevoo *et al.* (7), has shown the inhibitory effect of *S. frutescens* extracts on glucocorticoid biosynthesis. These data provide scientific evidence for the efficacy of *S. frutescens* as treatment of stress related illnesses.

In this study, the first question approached concerned the possible interaction of the plant material with the P450 enzymes. The difference spectra obtained confirmed that the *S. frutescens* extracts could interact directly with the cytochrome P450 enzymes. The resulting effect of this interaction was evident as these extracts could in part inhibit steroid substrate binding to the cytochrome P450 enzymes.

The second question that was approached, was whether the various *S. frutescens* extracts would influence the metabolism of the steroid substrates. In adrenal steroidogenesis CYP21 catalyses the hydroxylation of C21 of PROG and 17-OH-PROG yielding 11-DOC and 11 deoxycortisol respectively, while CYP17 stand at a branch point in steroidogenesis and catalyses the hydroxylation and lyase reactions necessary for the biosynthesis of cortisol and androgens respectively. The various *S. frutescens* extracts were able to influence the catalytic activities of the P450 enzymes. The inhibitory effect of the *S. frutescens* extracts on the CYP21 was higher than that of CYP17, influencing the flux of the cortisol precursor pathway. In the mitochondrial preparation, CYP11B1 catalyzes the 11β-hydroxylation of 11-DOC and
11-deoxycortisol to produce corticosterone and cortisol respectively. The *S. frutescens* extracts were also able to inhibit the catalytic activity of this enzyme. These results clearly indicate that the plant extracts can influence the biosynthesis of cortisol during adrenal steroidogenesis.

It is evident that the various *S. frutescens* extracts contains bioactive compounds which inhibited the steroidogenic P450 enzymes and thus can subsequently lead to the reduction of cortisol biosynthesis. The data obtained in this study, justifies the use of *S. frutescens* for the treatment of stress and diseases related to excessive blood cortisol levels such as Cushing’s syndrome. However, this plant might not be used as a replacement for conventional medicines but rather as an additional aid for the body to mobilize its own resources to cope with various physical and mental stresses, hence restoring a state of well-being.

In future, further studies will include further fractionating *S. frutescens* extracts in order to determine which bioactive compounds are responsible for influencing the catalytic activities of the P450 enzymes.
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