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

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 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.

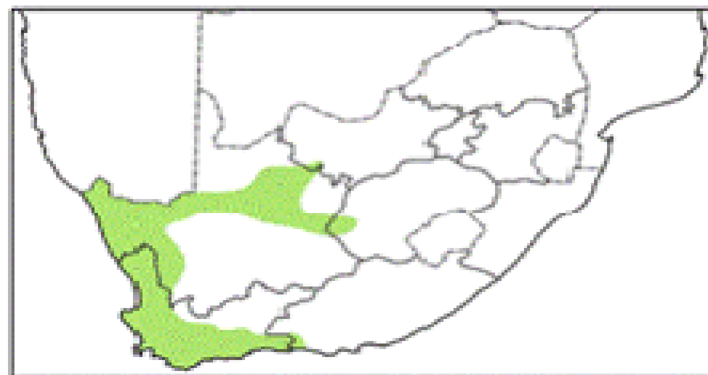

Figure 1. The shaded area, marks the geographical distribution of *S. frutescens* in the Western, Eastern and Northern Cape provinces of South Africa.

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.

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 ^{O}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

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.

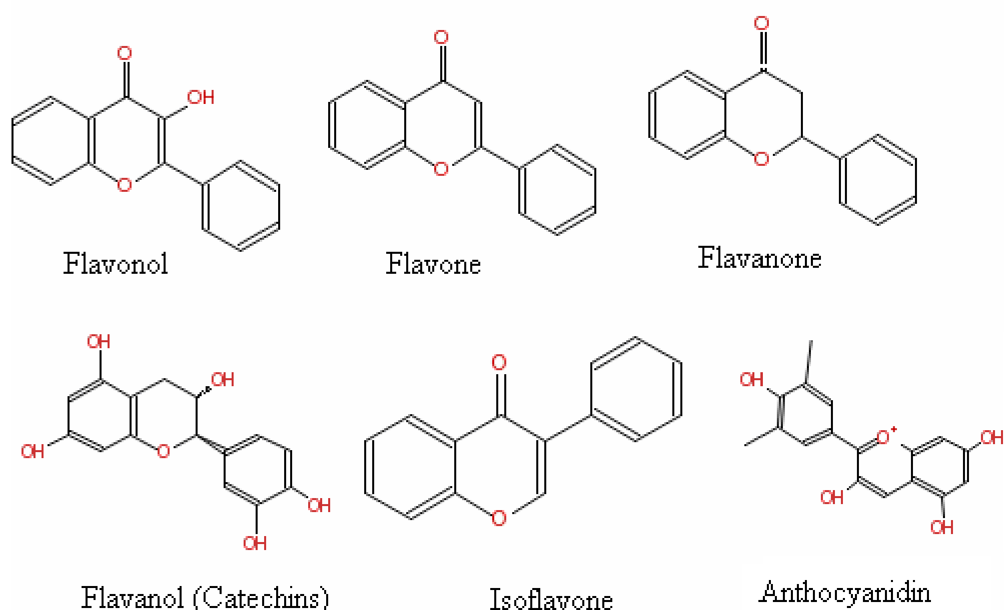

Figure 3. Flavonoids identified in medicinal plants.

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.

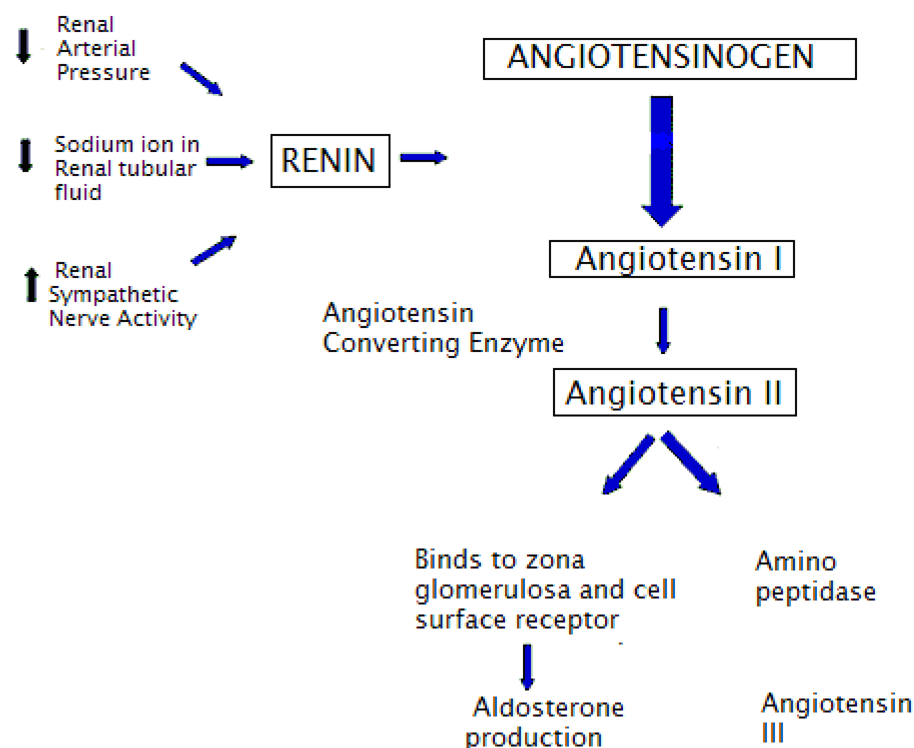

Figure 9. Schematic representation of the regulation of aldosterone secretion.

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).

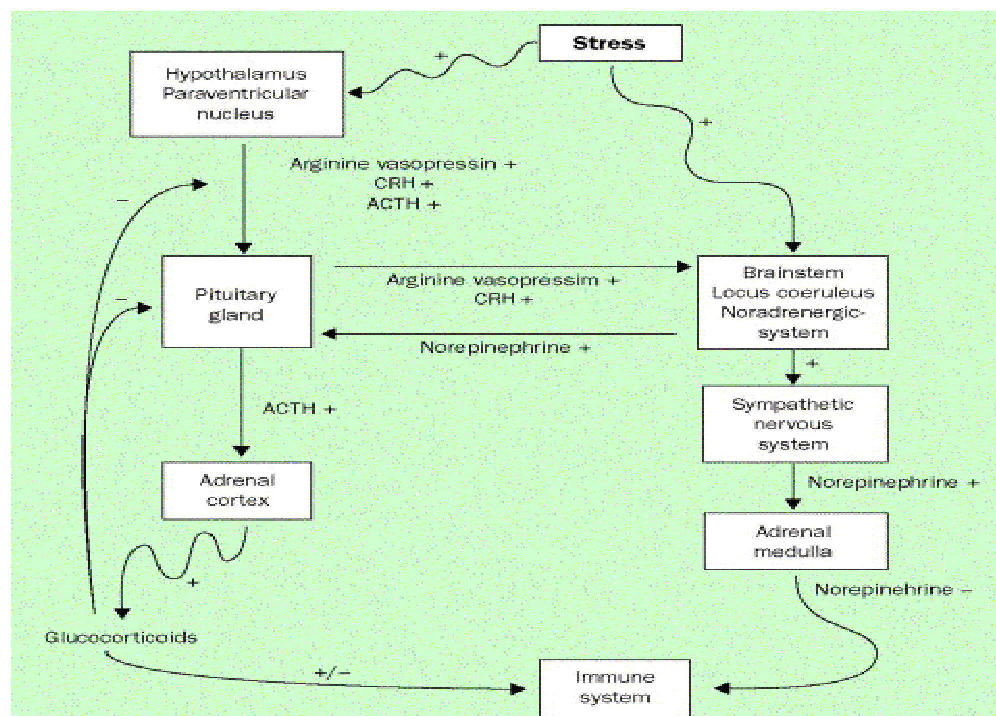

Figure 10. Schematic representation of glucocorticoid synthesis by adrenal cortex in stress response. Note ACTH and CRH secretion is inhibited by glucocorticoid feedback (130).

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.

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).

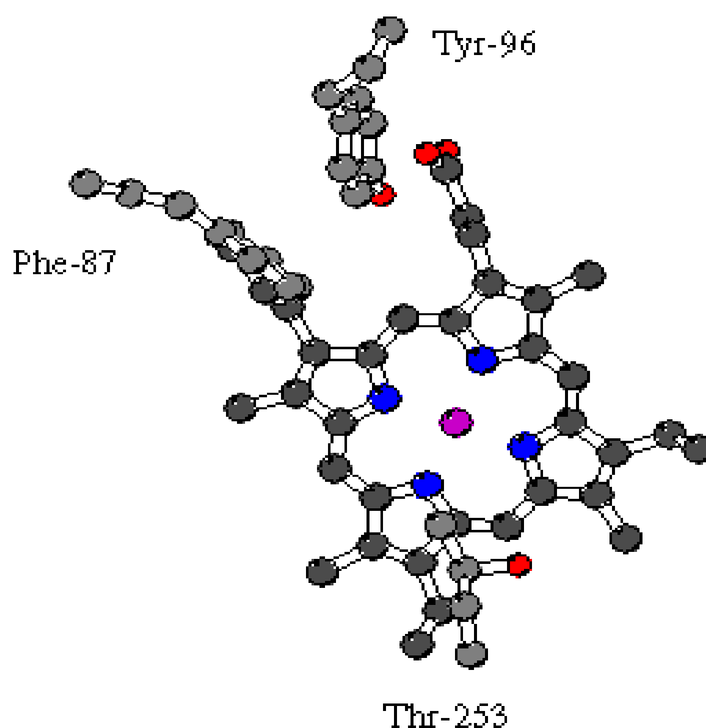

Figure 16 Active site of substrate free P450cam. Oxygen atoms are shown in red, nitrogen in blue, sulphur in grey and iron in purple. Carbon atoms are shown in black while hydrogen is not shown (182).

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

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.

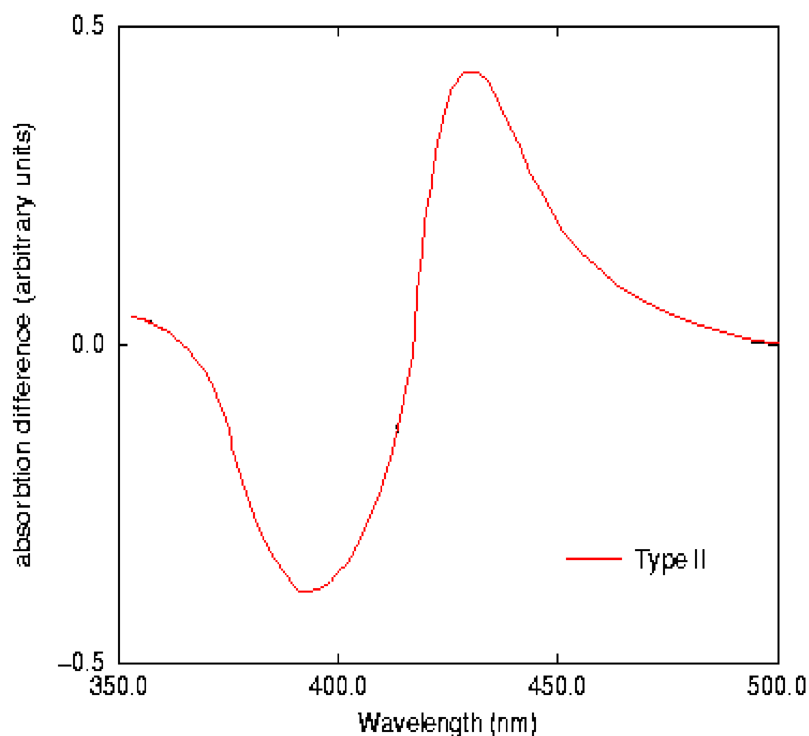

Figure 19. Type II difference spectra obtained by the addition of an inhibitor to cytochrome P450 (190).

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.

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.

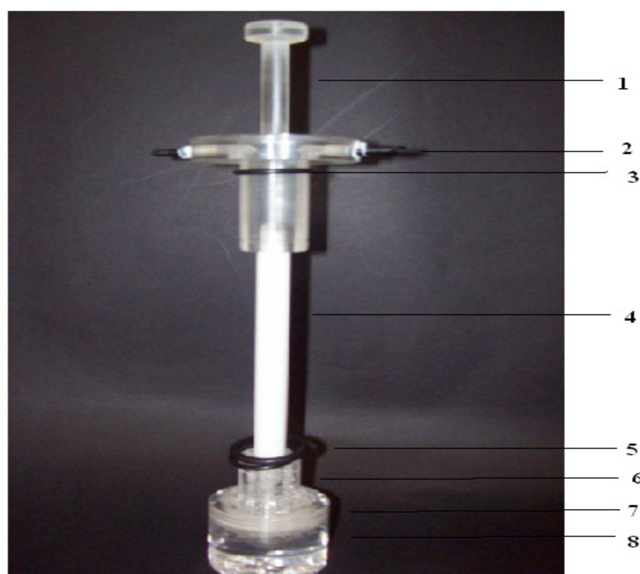

Figure 26. Assembled perspex apparatus constructed for concentrating large volumes of dilute protein solution. (1) top membrane support fitting, (2) sample inlet, (3) O-ring, (4) central spacing rod, (5) O-ring, (6) clamps, (7) bottom membrane support fitting and (8) sample collecting chamber

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.

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.

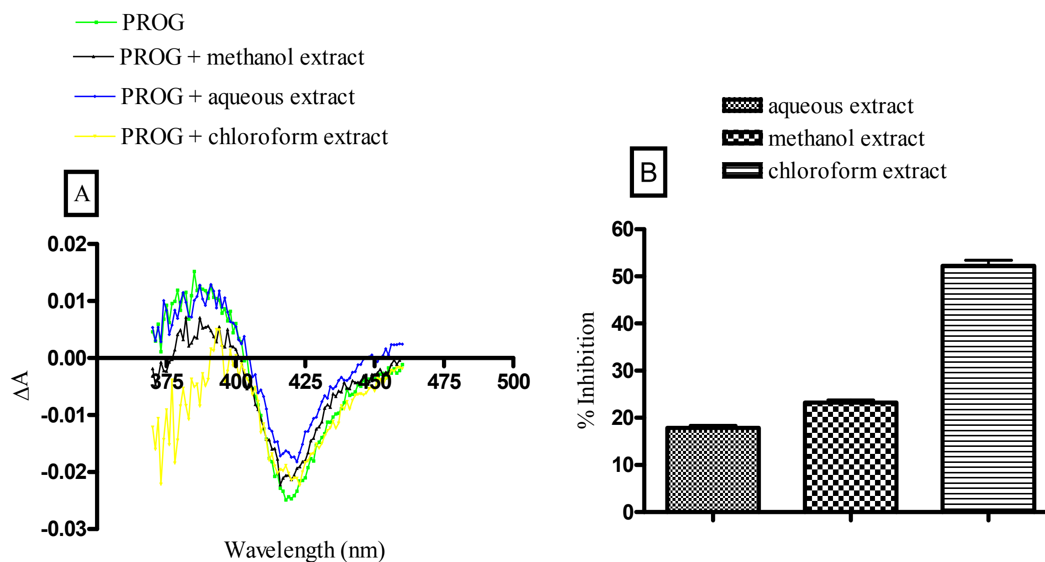

Figure 31. A: Inhibition of PROG-induced type I difference spectra in adrenal microsomes [cytochrome P450] = 0.7 µM, [PROG] = 32 µM ( ), aqueous extract, 1.06 mg ( ), methanol extract, 1.06 mg ( ) and chloroform extract, 1.06 mg ( ). B: Percentage inhibition of PROG binding to microsomal P450 enzyme 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.

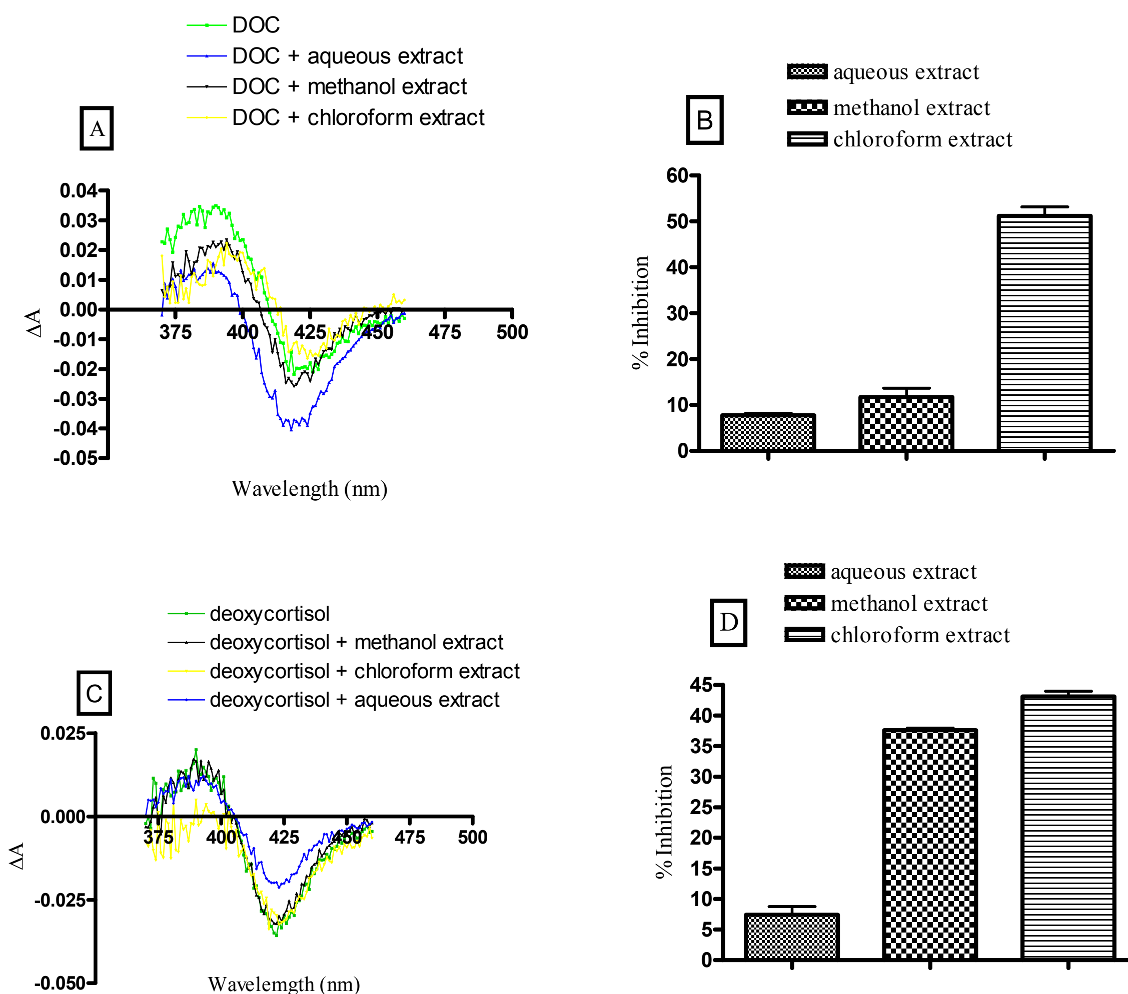

Figure 32. Inhibition of steroid-induced type I difference spectra by *S. frutescens* extracts in adrenal mitochondrial preparation. A: Inhibition of DOC-induced difference spectra and B: Percentage inhibition of DOC binding to CYP11B1, [DOC], 32 µM ( ); C: Inhibition of deoxycortisol-induced difference 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

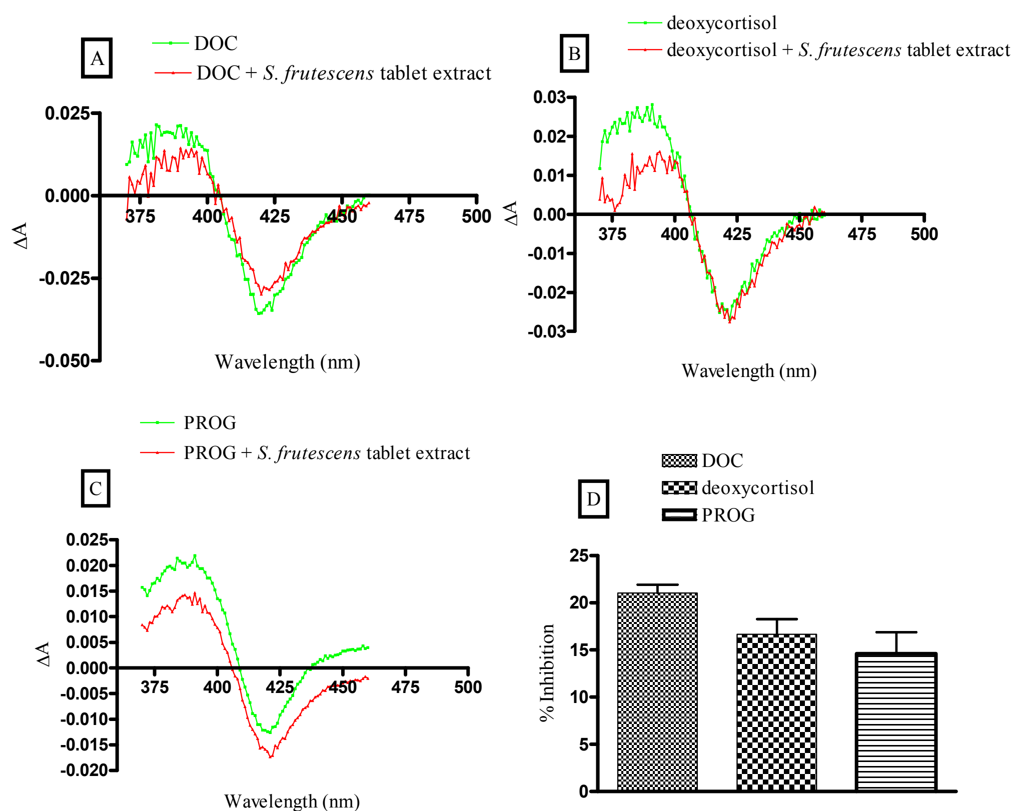

Figure 33. Inhibition of substrate-induced type I difference spectra in ovine adrenal mitochondria [Cytochrome P450] =0.7 µM, A: [DOC] = ( ) 32 µM. B: [deoxycortisol] = ( ) 32 µM. C: [PROG] = ( ) 32 µM. *S. frutescens* tablets extract, 1.06 mg ( ). D: Percentage inhibition of DOC, deoxycortisol and PROG 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.

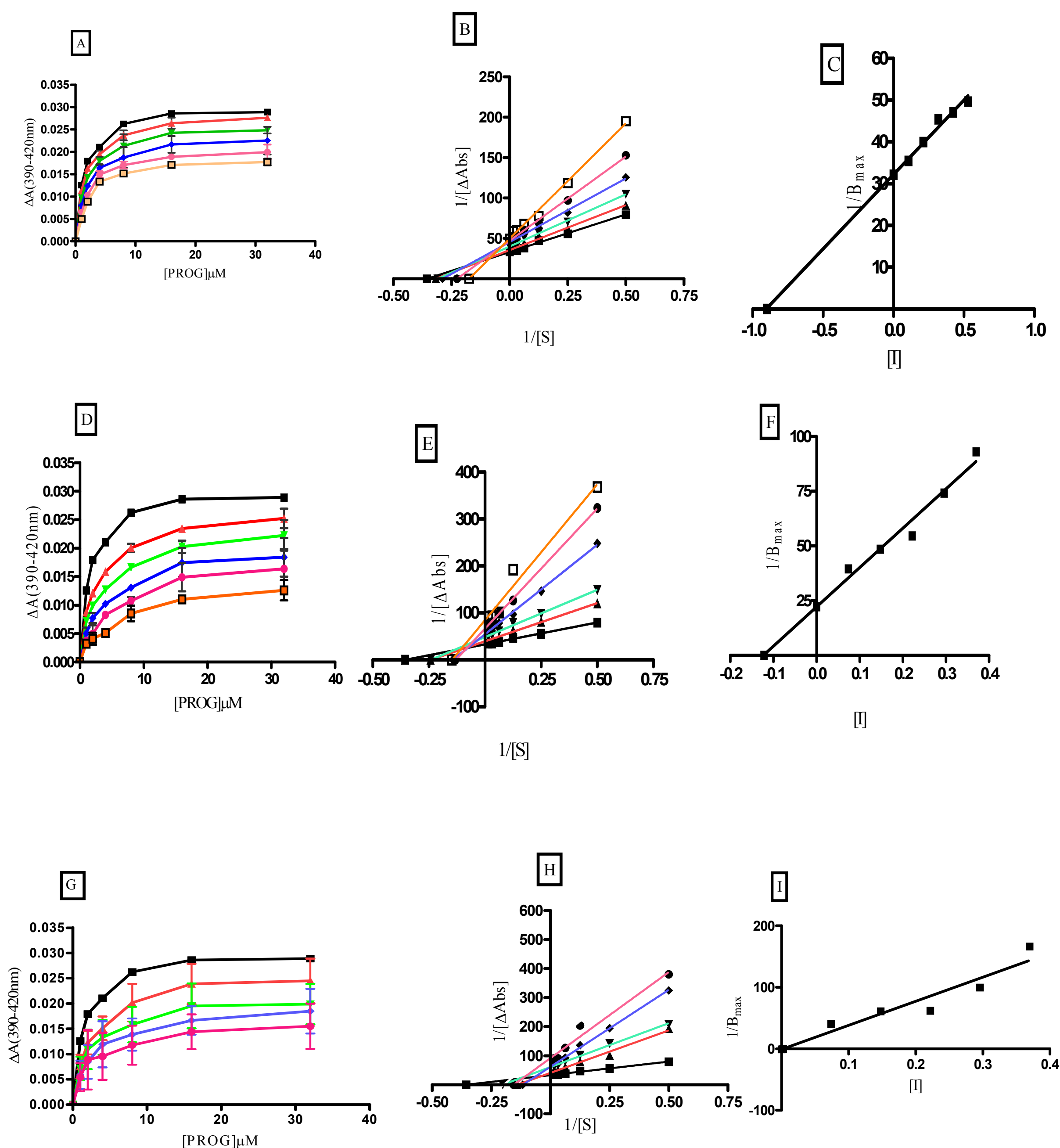

Figure 34. Hyperbolic saturation binding curves of the inhibitory effect of *S. frutescens* extracts on PROG binding to microsomal P450 enzymes: A: aqueous extracts; D: methanol extracts; and G chloroform extract. Double reciprocal plots of the inhibitory effect of *S. frutescens* extracts on PROG binding: B: aqueous extracts E: methanol extracts; H: chloroform extracts. Secondary plot of the slope of 1/ [ΔAbs] 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 *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