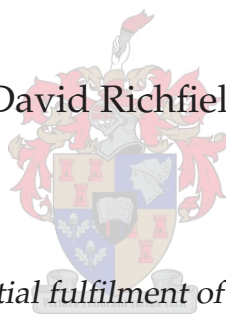UNIVERSITEIT • STELLENBOSCH • UNIVERSITY

An investigation into the biological activity of rooibos (*Aspalathus linearis*) extracts

by

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Biochemistry at the University of Stellenbosch

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Declaration

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

Signature: . . . .
            D. Richfield

Date: . . . .

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Summary

This study describes:

1. The preparation of chloroform, methanol and aqueous extracts of unfermented and fermented rooibos (*Aspalathus linearis*).

2. The chromatographic fractionation of aqueous rooibos extracts and an investigation into the polyphenol content and antioxidant activity of the fractions.

3. The preparation of ovine adrenal microsomes containing active steroidogenic P450 enzymes, including cytochrome P450 17α-hydroxylase, CYP17, and cytochrome P450 steroid 21-hydroxylase, CYP21.

4. An investigation into the influence of chloroform and methanol extracts of rooibos on the binding of steroid substrates, progesterone and 17-hydroxyprogesterone, to CYP17 and CYP21.

Opsomming

Hierdie studie beskryf:

1. Die voorbereiding van chloroform-, metanol- en waterekstrakte van ongefermenteerde en gefermenteerde rooibos (*Aspalathus linearis*).

2. Die chromatografiese fraksionering van water ekstrakte van rooibos en 'n ondersoek na die polifenolinhoud en antioksidantkapasiteit van die fraksies.

3. Die voorbereiding van skaapbyniermikrosome met aktiewe steroidogeniese sitochroom P450-afhanklike ensieme, onder andere sitochroom P450 17α-hidroksilase, CYP17, en sitochroom P450 steroïed 21-hidroksilase, CYP21.

4. 'n Ondersoek na die invloed van chloroform- en metanol-rooibosekstrakte op die binding van die steroïedsubstrate, progesteroon en 17-hidroksi-progesteroon, aan CYP17 en CYP21.

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- The helpful, professional and friendly technical and administrative staff of the Stellenbosch Biochemistry department.

Dedication

*To Surice, who had a part-time husband
for far too long.*

Contents

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Abbreviations and symbols

<div align="center">———— **General** ————</div>

2-AAF	2-Acetylaminofluorene
AGE	Advanced glycation end-product
B(a)P	Benzo[*a*]pyrene
BaP-7,8-OH	Benzo[*a*]pyrene-7,8-dihydrodiol
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
CAH	Congenital Adrenal Hyperplasia
CHP	Cumolhydroperoxide
CNS	Central Nervous System
CSF	Cerebro-spinal fluid
DPPH	α, α-Diphenyl-β-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmatic Reticulum
ESMS	Electrospray mass spectrometry
GAE	Gallic Acid Equivalents
GC/MS	Gas Chromatography/Mass Spectrometry
HPA	Hypothalamic-Pituitary-Adrenal
HPLC	High-performance liquid chromatography
HPLC-DAD	High Performance Liquid Chromatography with Diode-Array-Detection

MDA	Malondialdehyde
MMC	Mitomycin C
MMS	Methyl methanesulfonate
N-OH-PhIP	2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PEG	Polyethylene Glycol
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
TBARS	Thiobarbituric acid reactive substance
TBHQ	Tertiary Butyl Hydroquinone
TPA	12-O-tetra-decanoylphorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris(hydroxymethyl)aminomethane Hydrochloride

―――――――― **Enzymes** ――――――――

3βHSD	3β-Hydroxysteroid dehydrogenase
4CL	4-Coumarate CoA ligase
C4H	Cinnamate-4-hydroxylase
CHS	Chalcone synthase
CYP11A1	Cytochrome P450 cholesterol side chain cleavage
CYP11B1	Cytochrome P450 11β-hydroxylase
CYP11B2	Aldosterone synthase
CYP17	Cytochrome P450 17α-hydroxylase/17,20 lyase
CYP1A2	P450 dependent monooxygenase 1A2
CYP21	Cytochrome P450 steroid 21-hydroxylase
PAL	Phenylalanine amino lyase
PO	Peroxidase
PPO	Polyphenol Oxidase

——————— **Hormones** ———————

17OH-P4	17-Hydroxyprogesterone
17OH-P5	17-hydroxypregnenolone
18-OHB	18-hydroxycorticosterone
A4	Androstenedione
ACTH	Adrenocorticotropin
ALDO	Aldosterone
B	Corticosterone
Chol	Cholesterol
CRF	Corticotropin Releasing Factor
CRH	Corticotropin Releasing Hormone
DHEA	Dehydroepiandrosterone
DOC	Deoxycorticosterone
F	Cortisol
FSH	Follicle Stimulating Hormone
hGH	Human Growth Hormone
LH	Luteinising Hormone
P4	Progesterone
P5	Pregnenolone
PRL	Prolactin
S	11-Deoxycortisol
TSH	Thyroid Stimulating Hormone

——————— **Mathematical symbols** ———————

A	Absorbance
B_{max}	Maximum substrate binding capacity of enzyme
B_{max}^{app}	Apparent maximum substrate binding capacity of enzyme

c	Concentration
ε	Extinction coefficient
k_1	Substrate binding rate constant
k_{-1}	Substrate dissociation rate constant
k_{cat}	Enzyme catalytic reaction rate constant
K_i	Uncompetitive binding inhibition constant
k_I	Competitive inhibitor binding rate constant
k_{-I}	Competitive inhibitor dissociation rate constant
K_M	Michaelis constant
K_M^{app}	Apparent Michaelis constant
K_s	Substrate dissociation constant
K_s^{app}	Substrate dissociation constant
K_{si}	Competitive binding inhibition constant
l	Optical path length
V_{max}	Maximum reaction rate
V_{max}^{app}	Apparent maximum reaction rate

Chapter 1

Introduction

Rooibos tea, a traditional indigenous South African herbal tea, is growing in popularity both locally and abroad. This is partly due to its pleasant flavour, and partly due to the health properties attributed to it. Rooibos tea does not contain caffeine, is lower in tannin than tea produced from *Camellia sinensis*, and has been shown to have high antioxidant capacity [1]. In South African traditional medicine, rooibos is recommended for a variety of stress-related conditions. Although the antioxidant and antimutagenic capacity of rooibos have been thoroughly studied [1–7], its effects on the endocrine system remain to be elucidated.

Besides fermented rooibos that has been processed in the traditional manner, so-called "green" rooibos, which has not been subjected to the "fermentation" process, has gained market share in recent years. Although it has been shown to have higher antioxidative capacity than traditional rooibos, the differences between the health properties of green and traditional rooibos are still under active investigation.

The aims of this study were:

- To investigate the bioactive properties of fermented and unfermented rooibos

- To prepare and fractionate fermented and unfermented rooibos extracts to investigate their antioxidant properties

- To investigate the correlation between the antioxidant capacity and polyphenol content of rooibos

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- To determine whether the putative stress-modulating properties of rooibos can be partly explained in terms of an interaction with the adrenal cytochrome P450 enzymes of the glucocorticoid biosynthesis pathway

The history and economic importance of rooibos, as well as the research into its beneficial effects, are summarized in Chapter 2. Rooibos is a uniquely South African agricultural product, and the export earnings of the industry support a significant workforce. Rooibos has a unique polyphenolic composition, including aspalathin, which has not been described in any other plant, and nothofagin, which has to date only been found in rooibos and *Nothofagus fusca*, the New Zealand red beech [8–10]. Different samples of rooibos tea contain different polyphenol profiles, due to differences in processing and also the genetic makeup and cultivation conditions of the plant [11, 12]. Long recognized for its lack of caffeine and low levels of tannins, rooibos has more recently become known as a rich source of antioxidants [1, 2, 13]. This has been cited as the reason for the anti-aging effects of rooibos that have been observed in animal studies [14]. Research into the antimutagenic properties of rooibos has also delivered positive results [3, 4, 7]. Anticarcinogenic effects can be due to a number of effects, many of which have been observed with different polyphenols. An important class of anticarcinogens exert their effects via antimutagenesis – a reduction in the frequency of genetic changes in an organism. A number of herbal products show antimutagenic activity, and rooibos is no exception, with a variety of different *in vitro* and *in vivo* experiments showing unequivocal results [7, 15, 16]. In addition recent *in vivo* experiments also clearly demonstrate the anticarcinogenic effects of rooibos. Interestingly, a number of these experiments indicate an effect that is mediated via the P450 enzyme systems of the liver [7].

In chapter 3, tannins, lignins and flavonoids, plant polyphenols derived from the shikimate pathway, are introduced in terms of their chemical characteristics, their biosynthesis and their biological significance to plants and animals. Polyphenols, especially those containing catechol groups (phenolic vicinal diols) have potent antioxidant, radical-scavenging and metal-chelating properties. These properties, as well as their ability to absorb ultraviolet light, make them essential to terrestrial plant life [17, 18]. They are also used as signalling molecules and precursors to structural polymers in plants. In animals, plant polyphenols can have a significant dietary impact [19]. Although not essential nutrients, they have wide-ranging effects on animal health [17, 20–22]. Since rooibos is known to contain a significant polyphenol fraction [8], an investigation into rooibos polyphenols was launched – rooibos extracts were prepared and fractionated by hydrophobic interaction chromatography. The antioxidant capacity and polyphenol content of the

fractions were determined, and the fractions were subjected to HPLC/DAD and ESMS analysis to identify and quantify their polyphenol constituents.

Chapter 4 presents a brief overview of P450 enzyme systems in terms of their nature and distribution, the reactions they catalyze, their catalytic cycle, and their significance in adrenal steroid metabolism. Adrenal steroidogenesis is also discussed with respect to its significance to the homeostatic feedback loops of the hypothalamic-pituitary-adrenal (HPA) axis. The P450 enzymes are a diverse superfamily of heme-dependent monooxygenases that are represented in all branches of life [23, 24]. P450 enzymes have unique spectral properties which allow spectrophotometric observation of substrate binding [25]. This is a distinct advantage in the investigation of their substrate affinity and in the identification of enzyme inhibitors. They were first described in the liver, where they catalyse a wide variety of reactions, including synthesis of bile acids and metabolism of xenobiotics [26]. One of the more important families of P450 enzymes catalyses reactions in the biosynthesis of steroid hormones [27]. The synthesis of glucocorticoids, mineralocorticoids and androgen precursors takes place in the adrenal cortex, under control of the hormones of the HPA axis [27]. The major human glucocorticoid, cortisol, is one of the most important hormones in the human response to physical and emotional stressors [26]. The relative activities of the various adrenal steroidogenic enzymes are therefore crucial to normal homeostasis – over- or underproduction of cortisol lies at the heart of a number of diseases [26]. The effects of natural products on adrenal steroidogenic enzymes is therefore a promising target for investigation.

In chapter 5, the central role of the P450 enzymes CYP17 and CYP21 in mammalian adrenal steroidogenesis is briefly discussed in terms of steroid substrate metabolism. The mathematical analysis of different classes of enzyme inhibition is introduced and applied to the investigation of the inhibitory effect of rooibos on these enzymes. The use of spectrophotometric substrate binding assays to determine enzyme inhibition parameters as well as the advantages and limitations of the method are discussed. Methods are outlined for the preparation of subcellular adrenal fractions containing P450 enzymes, and for the subsequent spectrophotometric assays which were carried out to determine the influence of rooibos extracts on substrate binding to P450 enzymes. The results of these assays are presented.

Chapter 6 presents an overview of the results obtained in this investigation into the bioactivity of rooibos and the conclusions which were drawn from the data, as well as a discussion of the relevance of the results to human health.

Chapter 2

Rooibos (*Aspalathus linearis*): A South African health drink

"Then it was, like a red bush in the cinders, slowly devoured."
— Madame Bovary (Gustave Flaubert)

2.1   Introduction

The rooibos plant (*Aspalathus linearis* (N.L.Burm.) R.Dahlgr.) is a flowering shrub in the family *Fabaceae* that is endemic to the Cedarberg region of the Western Cape province of South Africa [2]. It is colloquially called rooibos (Afrikaans for "red bush") due to the red colour of the dried leaves.

Rooibos tea is an infusion made by steeping leaves and stalks in boiling water. Technically it is a tisane, or herbal tea as the term "tea" refers only to infusions made from *Camellia sinensis* (L.) Kuntze. As a traditional beverage rooibos tea, or "rooi tee", has enjoyed popularity in South Africa since the beginning of the 20th century [28].

Commercial production started in 1904 [29] and today, rooibos is an important commercial crop, with the industry employing in excess of 4 500 people. Approximately 30 000 ha of agricultural land is currently under rooibos cultivation, compared to 14 000 ha in 1991. The bulk of the crop (60%), with a

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net worth of R100 million, is exported to Germany, Japan, the Netherlands, the UK, Malaysia, South Korea and the USA [30].

Rooibos tea is held in high regard for its perceived health-promoting properties. Besides containing very low levels of tannins and no caffeine [13], it acts as an anti-oxidant [1, 5] and has antimutagenic properties [4]. It is mainly effective against indirect-acting mutagens – those that require activation by P450 enzymes, an effect which will be discussed in more detail below [3, 31]. Rooibos stimulates the immune system [32], and has been claimed to be effective in the treatment of a range of diseases, including hypertension, allergies, skin diseases, colic in infants, diabetes, liver diseases, insomnia and other sleep disorders, headaches, irritability, tension and mild depression [33, 34]. Although these claims have not been clinically investigated and are based on anecdotal evidence and folk wisdom, many are symptomatic of endocrine disorders that may possibly be precipitated by dysregulations of the stress response.

2.2 Bioactive compounds in rooibos

Rooibos tea is commercially produced from the "Rocklands" variety of rooibos, and is subjected to a so-called "fermentation[1]" process after harvesting. The plant material is bruised and allowed to undergo oxidation in the open air to develop its characteristic colour, aroma and flavour [35]. Rooibos which has not undergone this fermentation process is referred to as "green" or unfermented rooibos.

The chemistry of the phenolic components of rooibos has been under investigation for nearly half a century. Seminal research done in the 1960s identified a range of phenolic constituents of rooibos tea [36–38]. The main phenolic compounds in commercial rooibos were shown to be aspalathin [5, 37] and nothofagin [12], two structurally similar C-linked dihydrochalcone glycosides. To date, rooibos is the only known source of aspalathin [8], and rooibos and the New Zealand red beech (*Nothofagus fusca*) are the only known sources of nothofagin [10]. Since the original investigations, researchers have optimized extraction systems for rooibos flavonoids [39] and have identified the major flavonoid components: flavones – orientin, isoorientin, vitexin, isovitexin, luteolin and chrysoeriol; flavonols – quercitrin, isoquercitrin and quercetin; and phenolic acids – paracoumaric acid, parahydroxybenzoic acid,

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[1]Although this is not technically a fermentation, as it is neither anaerobic nor mediated by micro-organisms, the term was adopted from traditional tea production and is used as such in the scientific and technical literature of rooibos.

ferulic acid, protocatechuic acid, vanillic acid and caffeic acid [8, 9, 12, 40]. There are striking differences in the distribution of phenolic constituents between different species in the genus *Aspalathus*, and even between different wild populations of *A. linearis*, with some populations completely lacking aspalathin [11].

The minor flavonoids are a group of structurally related phenolic compounds including flavanones, flavanonols, chalcones, retrochalcones and dihydrochalcones. They are referred to as "minor" flavonoids because they only occur in significant quantities in a limited number of foods. Flavononols and retrochalcones do not occur in significant quantities in typical human diets. The main dietary source of flavanones in western diets is probably citrus, and the major sources of dihydrochalcones are probably apples and apple juice products, which include ciders [19].

In the fermentation process, polyphenolic compounds, most notably aspalathin ($2', 3, 4, 4', 6'$-pentahydroxy-3-C-β-d-glucopyranosyldihydrochalcone) and nothofagin ($2', 3, 4', 6'$-tetrahydroxy-3-C-β-d-glucopyranosyldihydrochalcone), are oxidised [12, 41]. This process is presumably mediated by the native enzymes polyphenol oxidase (PPO) and peroxidase (PO) [35]. A consequence of the fermentation process is that green rooibos has a higher antioxidative capacity than "fermented" rooibos [6].

Besides its phenolic components, rooibos contains a range of volatile components which are responsible for the rich aroma of rooibos, especially of the "fermented" product. As is the case with many natural substances, rooibos contains a complex mixture of chemical species. Analyses of organic extracts and steam distillates of rooibos extracts using gas chromatography and mass spectrometry (GC/MS) have identified paraffins, alcohols, aldehydes, ketones, acids, esters, lactones, imides, phenols and furans [42].

Bioactivity of rooibos

Antimutagenic and anticarcinogenic activity

A distinction must be made between antimutagenesis and anticarcinogenesis: antimutagenesis is the prevention of mutations, while anticarcinogenesis includes any effect that prevents the formation of cancers or reduces the

severity of existing cancers. Antimutagenic effects tend to be anticarcinogenic, since mutations can lead to the formation of cancer by activating proto-oncogenes or silencing tumor-suppressing genes [43]. Anticarcinogenic effects can arise due to increased apoptosis, inhibition of angiogenesis, inhibition of enzymatic activation of carcinogens (especially by the heme monooxygenases in the liver), the activation of endogenous carcinogen-deactivation systems [44–46], or stimulation of the immune response [32].

Polyphenolic compounds in general exert a variety of anticarcinogenic effects, including antiangiogenic activity [47], the inhibition of telomerases in cancer cells [48], promotion of apoptosis [49, 50], and stimulation of immune function [51]. A significant research effort, starting in 1988, has demonstrated the antimutagenic properties of polyphenols in teas made from *Camellia sinensis* [52], both green tea (unfermented) [53–55] and black tea (fully fermented) [52], as well as partially fermented teas like Oolong tea and po-lei (pu-erh) tea [1, 15]. Antimutagenic activity has also been demonstrated in herbal infusions such as tochu tea (*Eucommia ulmoides*) [56], lime flower (*Tilia cordata*) [57], verveine (*Lippia citriodora*) [57], mint (*Mentha × piperita*) [57], rosehips (*Rosa canina* fruit) [57] and nettles (*Urtica dioïca*) [57], as well as other beverages made from plant material, including coffee and cocoa [58].

Rooibos has been shown to exhibit antimutagenic activity, an effect that can be stronger in "fermented" or "unfermented" rooibos depending on the conditions and the type of assay used [3, 4]. In 2000, Marnewick *et al.* [3] demonstrated, using a salmonella mutagenesis assay, that rooibos and *Cyclopia genistoides* (honeybush) reduce the effects of indirect-acting mutagens 2- acetylaminofluorene (2-AAF) and aflatoxin B_1 (AFB_1) while being much less effective against the direct-acting mutagens methyl methanesulfonate (MMS), cumolhydroperoxide (CHP), and hydrogen peroxide (H_2O_2).

Although microbiological mutation assays have the advantage of being stable, relatively simple and cost effective, these assays are only partially indicative of mutagenic processes in mammalian tissues. A number of cell lines are, however, available with stably transfected cytochrome P450 monooxygenase enzymes [59], leading to the development of mutagenicity assays which more closely mimic the eukaryotic cellular environment. In one such assay, using a mutagenic challenge of 2-AAF, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), benzo[*a*]pyrene-7,8-dihydrodiol (BaP-7,8-OH) and 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) against a Chinese hamster lung fibroblast cell line engineered to express P450 dependent monooxygenase 1A2 (CYP1A2), rooibos was shown to have antimutagenic properties, more strongly against the promutagens AAF and PhIP than against the direct mutagens BaP-7,8-OH and N-OH-PhIP [31].

Marnewick *et al.* [7] investigated the effects of ethanol/acetone soluble fractions of rooibos methanol extracts in an *in vivo* mouse skin tumor model in 2005. They found strong metabolic antioxidant effects, as demonstrated by inhibition of the formation of thiobarbituric acid reactive substances (TBARS), as well as potent inhibitory effects against 12-O-tetra-decanoylphorbol-13-acetate (TPA) tumor promotion, especially by unfermented rooibos extracts. Green tea ethanol/acetone fractions were, however, even more effective.

These results would seem to indicate that at least one major effect of rooibos on mutagenesis is the inhibition of liver cytochrome P450 monooxygenases, leading to reduced activation of indirect-acting mutagens (promutagens). However, in 1993 Sasaki *et al.* [15] demonstrated anticlastogenic activity for rooibos against mitomycin C (MMC) and benzo[*a*]pyrene (B(a)P), both in the presence and absence of P450 enzymes, in contrast to green and po-lei teas, which mainly showed activity in the presence of P450 enzymes. No direct conclusions can be drawn from this study regarding the mechanism of rooibos's antimutagenic effect, however, as different mutagens were used.

In a rat model, rooibos and honeybush significantly enhanced the activity of cytosolic glutathione S-transferase alpha, a phase II drug metabolizing enzyme, but did not affect the oxidative status in the liver, unlike *Camellia sinensis*, which reduced the antioxidant capacity of the liver [60]. That no change in the oxidative status was seen with administration of rooibos over the short term is not unexpected, as polyphenols only tend to improve antioxidant status *in vivo* under conditions of oxidative stress [61]

Another intriguing result reported is the protective effect of rooibos against radiation-induced clastogenic effects [62], an effect that was not observed with green tea. As the mechanism of this effect is not yet understood, it is not clear which components of the tea might be responsible.

Antioxidant activity

Although many foodstuffs contain antioxidants as endogenous factors, the shelf life of processed foods can be extended by increasing their antioxidant capacity. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) can be used for this purpose, as they delay rancidification by inhibiting fatty acid oxidation. Commercial food producers have therefore included these compounds in a wide range of products since the 1950s [63], creating new possibilities for supply, transport and storage of foodstuffs.

In the last few decades, however, concern about the safety of food additives has increased, with synthetic antioxidants also coming under renewed investigation. Epidemiological studies have found no clear evidence that these compounds pose a risk to human health [64]. With some studies even finding anticarcinogenic effects, the US Food and Drug Administration [63] has classified BHA, BHT and THBQ as "generally accepted as safe" (GRAS) at the concentrations normally used in processed foods. Some animal studies on synthetic antioxidants have, however, reported carcinogenic effects [65, 66], and the national toxicology program of the US department of health has identified BHA as a substance "reasonably anticipated to be a human carcinogen" [67]. The fact that the animal and *in vitro* results are not all in agreement with each other or with the epidemiological studies shows that more research is warranted.

Since the public has become aware of these safety issues and of the advantages of antioxidants in foods, food supplements and cosmetics, an intensive research effort has focussed on the use of natural substances for their antioxidant properties, both in foods and for other purposes. Many plant materials show antioxidant activity, as they contain phenolic compounds that act as primary antioxidants, which can terminate the chain reaction of lipid peroxidation [68]. Among the natural additives commercially produced for use in the production of processed foods are herb extracts, e.g. rosemary and sage, as well as tea extracts [69]. The antioxidant activity of rooibos has been subjected to much scrutiny since von Gadow *et al.* [1] showed in 1996 that it has higher α, α- diphenyl -β- picrylhydrazyl (DPPH) radical scavenging activity than black or oolong tea. Furthermore it was shown in 1997 that aspalathin has a comparable antioxidant activity to α-tocopherol, BHA and BHT [5].

The formation of AGEs (advanced glycation end-products), a process which is intimately associated with conditions of oxidative stress [70], the formation of TBARS and the development of vascular and diabetic diseases [71], has also been shown to be suppressed by rooibos *in vitro* [72]. This is most likely due to the antioxidant effects of flavonoids identified in rooibos. Quercetin, amongst other flavonoids, has been shown to inhibit lipid peroxidation in macrophages by delaying the depletion of endogenous α-tocopherol [20]. In addition, the induction of oxidative stress by AGE albumin in normal animals can be prevented by pretreatment with antioxidants [70].

Ad libitum administration of rooibos to rats almost completely suppresses the age-related accumulation of lipid oxidation products in the central nervous system (CNS) [14]. In a rat model where a diabetic state was induced with streptozotocin, rooibos partially prevented oxidative stress, as demonstrated by a decrease in AGEs and malondialdehyde (MDA) [73]. Similar results were also achieved in a rat model where liver damage was induced by CCl_4

administration – levels of α-tocopherol and the reduced form of coenzyme Q_9 (CoQ_9H_2) were restored by rooibos [74], further confirming that flavonoids in rooibos exert at least some of their biological effects by replenishing native antioxidant species in the body.

2.3   Summary

It is evident that rooibos is an important commercial crop in South Africa, with a significant economic contribution to rural communities. Its reputation as a wholesome, healthy drink has helped it to secure a position in the international herbal tea market – a position which was strengthened by the recent legal settlement whereby international trademarks that had been registered for "Rooibos" by Forever Young were cancelled [75]. The expansion of knowledge of the medical benefits of rooibos is therefore significant to the continuing effort to market this uniquely South African tea.

Although the claims surrounding rooibos have to an extent been supported by the research into its antioxidant properties, much remains to be learned about rooibos, both in terms of its minor constituents and in terms of its effects on the different homeostatic systems of the body.

An investigation into the antioxidant properties of rooibos will be discussed in the following chapter. The fractionation of rooibos extracts and subsequent HPLC and mass spectrometric analysis allowed the characterization of the polyphenol content in terms of the major and minor polyphenol compounds contributing towards the antioxidant capacity of rooibos.

Chapter 3

A preliminary investigation of the polyphenols in and antioxidative properties of rooibos

"I am now persuaded that my first supply was impure, and that it was that unknown impurity which lent efficacy to the draught."
— The Strange Case of Dr. Jekyll and Mr. Hyde (Robert Louis Stevenson)

3.1   Introduction

Although rooibos contains a complex mixture of chemical compounds, many of which have been linked to its health promoting properties, its anti-oxidant characteristics are believed to be largely due to its polyphenolic components, i.e. those compounds with more than one phenol group. Polyphenols in plants have a range of positive influences on human health, including cyto-protective and anticarcinogenic effects, as discussed in chapter 2.

The special characteristics of phenolic hydroxyl groups, especially in the catechol or vicinal diphenol configuration, are crucial to the biochemical effects of polyphenols. They exert their influence at cellular level partly by virtue of their antioxidant and radical-scavenging activity [17, 18], and partly due

to their ability to chelate transitional metal ions that initiate fatty acid oxidation [18]. There is also evidence that some of their positive effects may be mediated via their interactions with enzymes and cellular receptors [76]. Polyphenols occur in almost all plants, as structural polymers, intra- and inter-organism signalling molecules, antioxidants and defensive agents against predation. They also function as ultraviolet screens, and, in the case of polyphenols with strong absorbance in the visible and UV spectrum, attract pollinators [76].

Plant polyphenols are synthesised from phosphoenolpyruvate and erythrose-4-phosphate in the shikimate pathway. They are synthesised from shikimic acid via phenylalanine or tyrosine, with some exceptions, notably gallic acid, which is also synthesised from other intermediates of the shikimate pathway [76]. Plant polyphenols that derive from shikimate are collectively referred to as phenylpropanoids. Tannins, lignins and flavonoids are three major classes of phenylpropanoids.

Tannins

The tannins are a heterogeneous class of compounds, made up of polymers and oligomers of polyphenols, and are subdivided into condensed, hydrolysable, and derived tannins [77].

The condensed tannins, or proanthocyanidins, are polymers and oligomers of flavan-3-ols, and are constitutively expressed in a wide range of plants. Doubly-linked dimers, linked by $4 \rightarrow 8$ C-C bonds and $2 \rightarrow 7$ C-O-C bonds are known as A-type tannins; singly-linked oligomers linked by $4 \rightarrow 8$ or $4 \rightarrow 6$ C-C bonds are known as B-type tannins when dimeric or C-type tannins when trimeric (fig. 3.1). The bulk of proanthocyanidins, however, are polymers with higher degrees of polymerization, and polymeric condensed tannins with degrees of polymerization up to 17 have been characterised. Proanthocyanidins are frequently glycosylated or acylated, usually with gallic acid [78].

Despite some recent progress, the mechanism of the proanthocyanidin condensation reaction has not as yet been elucidated [79], and although the 2R,3R-2,3-*cis* conformation predominates in proanthocyanidins, there is still debate over whether it is an enzymatically mediated process [80]. It is postulated to proceed via a quinone methide intermediate [80, 81], but this remains to be confirmed.
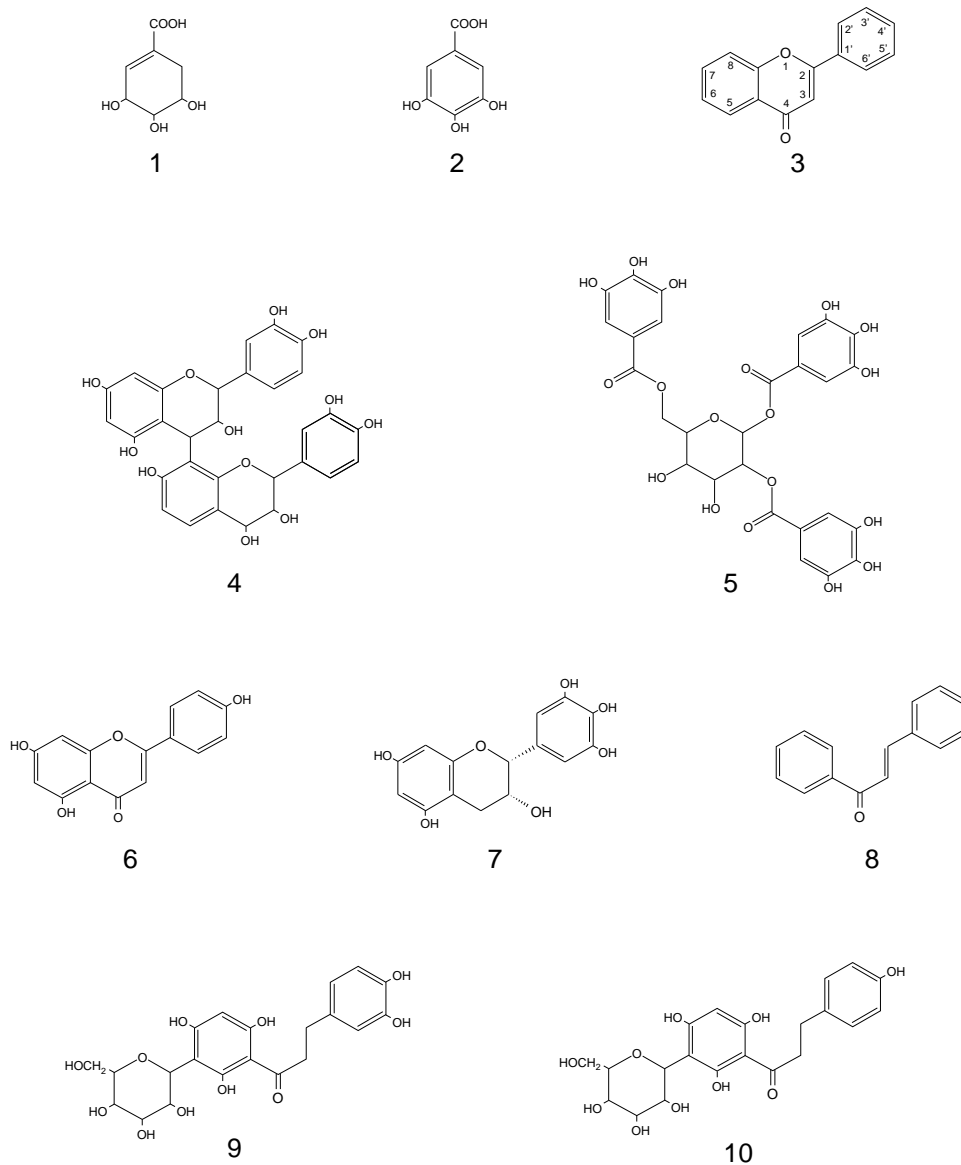
Figure 3.1: Polyphenol structures: **(1)** shikimic acid, a polyphenol precursor; **(2)** gallic acid, a phenolic acid; **(3)** general flavonoid structure; **(4)** procyanidin B, a condensed tannin; **(5)** trigalloyl glucose, a hydrolysable tannin; **(6)** genistein, an isoflavone; **(7)** epigallocatechin, a flavan-3-ol; **(8)** chalcone; **(9)** aspalathin, a dihydrochalcone; and **(10)** nothofagin, a dihydrochalcone;

Hydrolysable tannins differ from condensed tannins in that they consist of phenolic acids linked to a central glycosidic core by ester bonds [82]. They do not undergo chain-lengthening, and therefore do not exist as polymers.

In contrast to the condensed and hydrolysable tannins, the derived tannins are mostly formed during the processing of foods, as in the fermentation of tea. These are complex polymers, and are not, to date, well characterised. Theaflavins and thearubigins are important constituents of tea made from *Camellia sinensis* [77], but are not significant constituents of rooibos tea.

Many flavonoid-rich plant products (notably tea made from *C. sinensis*) contain high levels of tannin. Rooibos tea, however, is well known to contain low levels of tannins [8, 13, 83]. This has been favourable to the development of the rooibos industry, as tannins have long been recognised as antinutritional factors which interfere with the absorption of proteins [84, 85] and minerals [86]. The fact that rooibos does not contain high levels of tannins is also significant in terms of its method of consumption: Because it develops astringency more slowly than *C. sinensis* tea, rooibos tea is brewed for extended periods of time.

Rooibos is not, however tannin-free. Procyanidin B and a trimeric profistinidin have been isolated from rooibos in small amounts [8], and in 1984 Joubert [87] reported that tannins made up 80% of rooibos tea flavonoids.

The conflicting reports of the tannin content of rooibos are presumably partly due to differences in the raw material used. The tannin content of plant material generally increases with the age of the tissue, with larger amounts of tannins also being expressed under conditions of physiological stress. The flavonoid extraction conditions might well be a more important source of variation. Tannins have a higher molecular weight than monomeric flavonoids, and hence have a lower diffusion rate through plant matter. Longer extraction times and higher extraction temperatures would therefore favour more efficient extraction of tannins [88]. This is a significant factor in the analysis of polyphenols since rooibos tea is traditionally subjected to long brewing times, as mentioned above.

The major rooibos flavonoids, the dihydrochalcones aspalathin and nothofagin (9 and 10, fig. 3.1), do not possess the flavan-3-ol structure that is characteristic of natural proanthocyanidins [12, 78]: although proanthocyanidins lacking a hydroxy group at the 3 position of any of the monomers do exist, they are rare [89]. In contrast, four major flavan-3-ols which include epigallocatechin (fig. 3.1) constitute about one third of the dry mass of green tea [90]. Rooibos flavonoids are also unusual in that they are C-glycosides, as opposed

to the O-glycosides that make up the bulk of known glycosylated proantho-cyanidins [78]. The possible influence of these structural features on the low levels of tannins seen in rooibos tea is attractive material for speculation, but until more is known about the formation of condensed tannins, no concrete conclusions can be drawn.

Lignins

Lignins, while also polymeric plant polyphenols, are fundamentally different from tannins. They are three-dimensional, highly cross-linked polymers, largely made up of oxidatively-linked hydroxy-cinnamoyl alcohols and carbohydrates such as hemicellulose. The function of lignin in plant tissue is primarily to increase the structural strength of the plant, providing protection against damage due to environmental factors and actions of herbivores [76].

Lignin, classified as dietary fibre, acts as an antinutritional factor in foods, inhibiting mechanical disruption of the plant material, preventing the absorption of digestible protein and carbohydrate, and binding amino acids and bile salts in the intestine. The influence of lignin in herbal extracts and teas on human health may depend on context, however, because although it has antioxidant effects and can bind certain carcinogenic substances it is practically insoluble due to its high molecular weight [91, 92].

Flavonoids

Flavonoids, ubiquitous in terrestrial vascular plants, are a large class of secondary metabolites derived from phenylpropanoid metabolism. Some important subgroups of flavonoids are the flavones, isoflavones, chalcones, flavonols and anthocyanins. Flavonoids are derivatives of 2-phenyl-4-benzopyrone (flavone), with phenolic hydroxyl or methoxyl substituents at positions $3 - 6$ and $3' - 5'$ of the two aromatic ring systems (fig. 3.1). These moieties are often glycosylated [20].

The expression of flavonoids is increased in response to physical, nutritional and thermal stress, as well as increased levels of light [93]. This increased expression is mediated, at least in part, by chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis [76]. CHS catalyzes the synthesis of naringenin chalcone from 4-coumaroyl coenzyme A and malonyl coenzyme A, the biosynthetic step which initiates flavonoid biosynthesis from phenylpropanoid intermediates (fig. 3.2).
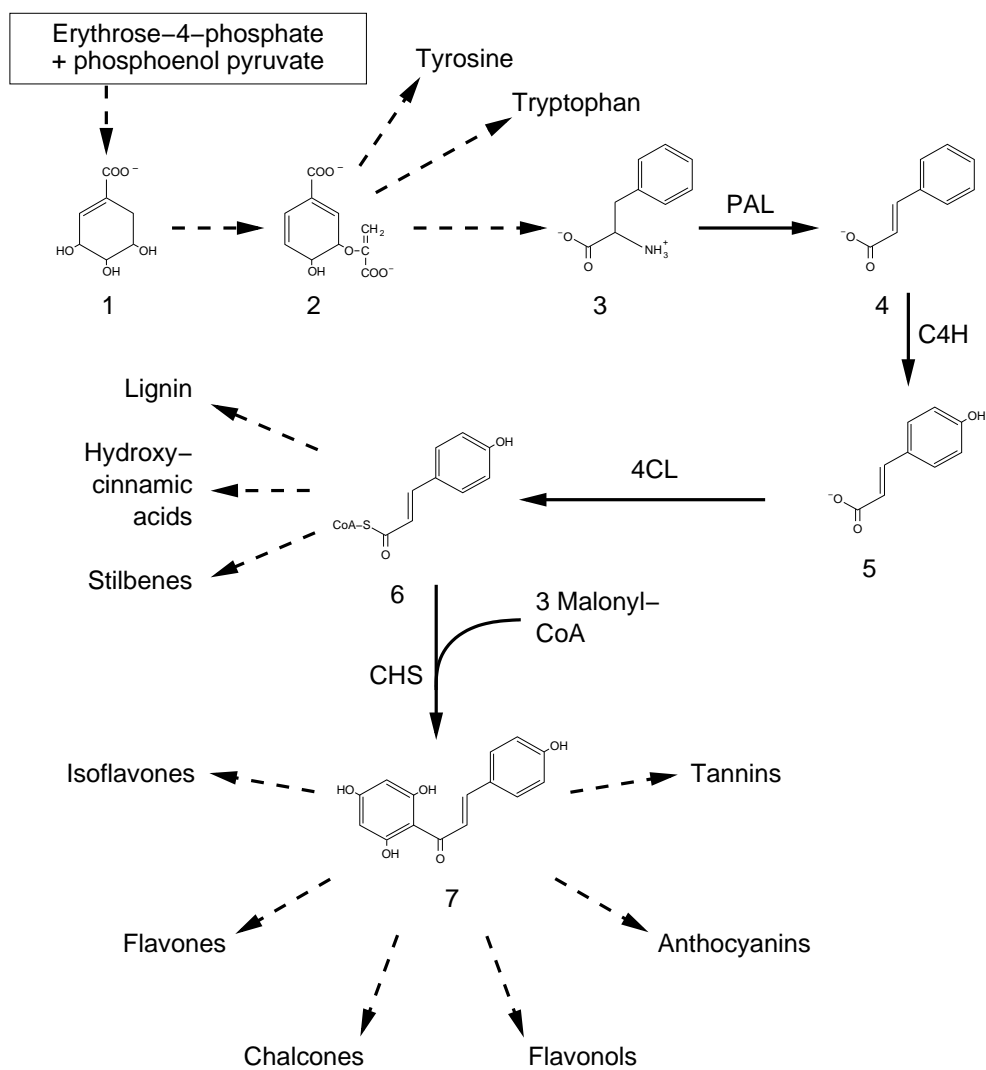
Figure 3.2: Flavonoid biosynthesis: (1) shikimate; **(2)** chorismate; **(3)** phenyl-alanine; **(4)** cinnamate; **(5)** 4-coumarate; **(6)** 4-coumaroyl coenzyme A; **(7)** narin-genin chalcone; **(PAL)** phenylalanine amino lyase; **(C4H)** cinnamate-4-hydroxylase; **(4CL)** 4-coumarate CoA ligase; and **(CHS)** chalcone synthase

Aside from their important role as substrates for tannin synthesis, flavonoids are highly efficient ultraviolet screens and, as such, are concentrated in plant tissues such as leaves and petals that are exposed to high levels of solar radiation. Some flavonoids, such as the anthocyanins, are brightly coloured, and act as signals to pollinators in petals and as signals to seed distributors in fruit. The antioxidant and free-radical scavenging properties of flavonoids are also crucial to the homeostasis of plants, especially under conditions of stress [76, 93].

The antioxidant and antimutagenic activity of aqueous rooibos extracts have been intensively investigated, and are largely attributed to the flavonoid constituents of rooibos [94]. Besides low levels of catechin and varying levels of flavonoid oligomers and polymers, as discussed above, rooibos contains high levels of C-linked flavonoid glycosides, including aspalathin, nothofagin, vitexin, isovitexin, orientin and isoorientin [9].

The antimutagenic activity of flavonoids has been linked to cytochrome P450-dependent enzyme systems. Rooibos flavonoids in particular exert biological effects that appear to involve the modulation of the activity of cytochrome P450 oxidase enzymes. This evidence comes from three lines of investigation: (i) the antimutagenic activity of rooibos extracts was enhanced in the presence of P450 enzyme isolates [3, 4, 6, 16] (ii) rooibos tea was effective in *in vivo* experiments using toxic challenges known to require activation by P450 enzymes [74] and (iii) flavonoids known to occur in rooibos have been shown to induce the expression of P450 enzymes [61]. However, little is known about the contributions of the different components of rooibos to the effects of rooibos extracts on cytochrome P450 enzymes.

A significant advance was made in this regard in 1996 by Shimoi *et al*, [22] who prepared fractions from an aqueous rooibos infusion by gel permeation / hydrophobic interaction chromatography using a Sephadex LH-20 adsorbent resin. A strong correlation was shown between the anticlastogenic activity and antioxidant capacity of the different fractions. In 2000, Marnewick *et al*. [3] compared methanol and aqueous extracts of unfermented rooibos with respect to the effects of on a range of mutagens in an *in vitro* antimutagenesis assay. The studies showed that the methanol extract was more effective against direct-acting mutagens and less effective against indirect-acting mutagens than the aqueous extract. In 2004, Joubert *et al*. [95] performed liquid-liquid extractions on an aqueous extract of rooibos which had been prepared at 100 °C, yielding aqueous and ethyl acetate soluble fractions. They showed a significant correlation between polyphenol content and radical scavenging activity, with the ethyl acetate fraction having a higher concentration of the active flavonoid constituents.

A variety of methods have been published for the analysis of phenolic compounds in foodstuffs [96–101]. Rooibos tea, however, being so rich in such a wide variety of closely related polyphenolic components, presents special challenges in the separation and identification of phenolic compounds. Apart from the known components of rooibos, a host of minor constituents exist that have as yet eluded identification. However, much progress has recently been made in LC/MS analysis of plant products, enabling simultaneous separation, identification and quantification [102]. Apart from the ubiquitous octadecyl silane HPLC methods used to resolve flavonoids and related compounds [103], a variety of normal-phase methods have also been devised for the separation of proanthocyanidins and other polyphenolic polymers and oligomers [104]

The identification and characterisation of bioactive compounds in rooibos would be facilitated by the preparation of rooibos fractions containing only certain groups of polyphenolic compounds. Apart from the challenge of characterisation, the fractionation of rooibos would greatly aid in the identification of the chemical species involved in its health-supporting effects. A proprietary method was therefore developed in cooperation with Benedict Technology Holdings for the preparation of rooibos fractions with differing hydropathic properties. Exploiting the specific characteristics of these fractions presented an opportunity to characterise them in terms of their polyphenol content, antioxidant capacity and biological activity.

3.2   Materials and methods

Materials and equipment

Aqueous extracts of fermented and unfermented rooibos (technical grade) were supplied by Benedict Technology Holdings (Pty) Ltd., South Africa. Aspalathin and nothofagin were purchased from the Medical Research Commission of South Africa, and 3,4 dihydroxybenzoic acid (DHBA, protocatechuic acid), caffeic acid, luteolin, vitexin, quercetin 3-β-D-glucoside, quercetin dihydrate, ferulic acid, rutin hydrate, syringic acid, vanillic acid and p-coumaric acid were purchased from Sigma-Aldrich (St Louis MO USA). Analytical standards were made up to a concentration of 1 mg/mL in ethanol or purified water. Reagents and solvents were purchased from Merck (Darmstadt, Germany).

High performance liquid chromatography (HPLC) chromatograms were recorded using a ThermoSep SpectraSystem consisting of a P4000 solvent delivery system, an AS3000 autosampler and column oven and a UV6000LP

diode-array detector. The system was controlled by a Windows™ 2000 workstation running ThermoQuest™ software [105]. Hydrophobic interaction chromatography (HIC) columns were supplied by Benedict Technology Holdings (Pty) Ltd., South Africa. Syringe filters were purchased from Microsep (Pty) Ltd., South Africa.

Electrospray mass spectrometry (ESMS) spectra were recorded using a Micromass triple quadrupole mass spectrometer fitted with an electrospray ionization source. The carrier solvent was 50% acetonitrile in water delivered at a flow rate of 20 μL/min. The sample solution (5 μL) was introduced into the ESMS using a Rheodyne injector valve. Data acquisition was done in negative mode, with a cone voltage of 60 V.

Processing of fermented and unfermented rooibos extracts

Immediately prior to use, the aqueous rooibos extracts were centrifuged at $7\,500 \times$ g for 30 minutes at room temperature. The supernatants were subsequently fractionated using two different HIC columns. Column effluent fractions of differing hydrophobicity were collected, dried under reduced pressure with a rotating evaporator (Büchi, Switzerland) and lyophilized prior to further analysis.

Immediately prior to use, lyophilized samples were redissolved in deionized water to a concentration of 1 mg/mL and centrifuged on a low-speed benchtop centrifuge. The insoluble pellet was discarded, and the supernatant was filtered using a 20 μm pore size filter.

Determination of polyphenol content and antioxidant capacity of rooibos fractions

Polyphenols in fermented and unfermented rooibos column fractions were determined as described by Singleton and Rossi [106]. Assays were carried out by ARC/Infruitec Nietvoorbij according to the Folin-Ciocalteu method using gallic acid as a standard. Briefly, the sample (200 μL) was mixed with 7.5% Na_2CO_3 (800 μL) and Folin-Ciocalteu's reagent (1000 μL) and incubated at $30\,°C$ for 90 minutes. The absorbance was subsequently measured at 765 nm. Results are expressed as % gallic acid equivalents (%GAE).

Antioxidant capacities of fermented and unfermented rooibos column fractions were determined by ARC/Infruitec Nietvoorbij according to the α, α-diphenyl-β-picrylhydrazyl (DPPH) scavenging method, by monitoring the decrease in the DPPH concentration at 515 nm, as described by Brand-Williams *et al.* [107].

Identification and quantification of rooibos polyphenols

Fermented and unfermented rooibos extracts and reconstituted HIC column fractions were subjected to HPLC with diode-array-detection (HPLC-DAD) to identify and quantify polyphenolic compounds. HPLC-DAD allowed for detection of substances with different UV absorbance maxima as well as peak identification by comparison of elution times and UV absorbance spectra with those of known standards.

Aspalathin, nothofagin, 3,4 dihydroxybenzoic acid, caffeic acid, luteolin, vitexin, quercetin 3-β-D-glucoside, quercetin dihydrate, ferulic acid, rutin hydrate, syringic acid, vanillic acid and *p*-coumaric acid standards were prepared and subjected to HPLC analysis. Retention times were determined and UV spectra were recorded with the diode-array detector. The background absorbance of the eluent was subtracted from the recorded spectra, and the spectra were saved as spectral reference library files for use in peak identification during subsequent runs. The recorded spectra are included in appendix B.

The wavelength of maximum absorbance was determined for each standard compound. Calibration curves were generated by injecting a range of volumes of known concentrations of the standards to allow quantitative determination of samples of unknown composition.

Once the calibration curves had been generated, the following samples were assayed: unfermented and fermented rooibos extracts, and HIC column fractions of intermediate hydrophobicity derived from each of the rooibos extracts.

Fresh rooibos tea extracts were filtered using 20 μm pore size syringe filters immediately prior to use. Analytical standards were made up to a concentration of 10 μg/mL in ethanol or purified water.

Reverse phase HPLC (RP-HPLC) was carried out using a Zorbax SB-C18 column, particle size 3.5 μm, 3.0$\times$150 mm under the following conditions, using a linear gradient:

Solvent A: 1% formic acid in water
Solvent B: 100% acetonitrile
Column temperature 35 °C

Time [min]	% A	Flow rate [mL/min]
0	100	0.5
4	100	0.5
25	75	0.5
30	40	0.5
30.1	40	0.7
35	100	0.7
40	100	0.7

Normal-phase HPLC (NP-HPLC) was carried out using a Supelcosil LC-SI HPLC column, 5μm particle size, 250×4.6 mm, under the following conditions using a linear gradient:

Solvent A: 96% Methanol, 2% Acetic acid, 2% H_2O
Solvent B: 84% Dichloromethane, 14% Methanol, 2% Acetic acid
Flow rate constant at 1 mL/min
Ambient temperature

Time [min]	% A
0	0.0
30	17.5
60	44.0
65	88.0
70	88.0
75	0.0
80	0.0

Chromatograms were recorded using a diode array detector, set to monitor the eluent over the wavelength range 200-400 nm.

3.3 Results and discussion

Analyses of polyphenol content and antioxidant capacity of rooibos fractions

Fermented and unfermented rooibos extracts were separated by HIC into two fractions – a hydrophilic fraction, which eluted earlier, and a hydrophobic fraction, which eluted later. The recovered fractions were lyophilized and subjected to assays for polyphenol content and antioxidant capacity. The hydrophobic fraction showed a substantial enrichment – in terms of both antioxidant capacity and polyphenol content (figures 3.3 and 3.4) compared to the hydrophilic fraction.

Interestingly, the data also shows a marked difference between the antioxidant capacity and polyphenol content of the hydrophilic fractions of the fermented and unfermented extracts. The antioxidant capacity and polyphenol content of the hydrophilic fraction of the fermented extracts was markedly higher than that of the unfermented extract. It was therefore decided to perform a more extensive fractionation of the rooibos extracts.
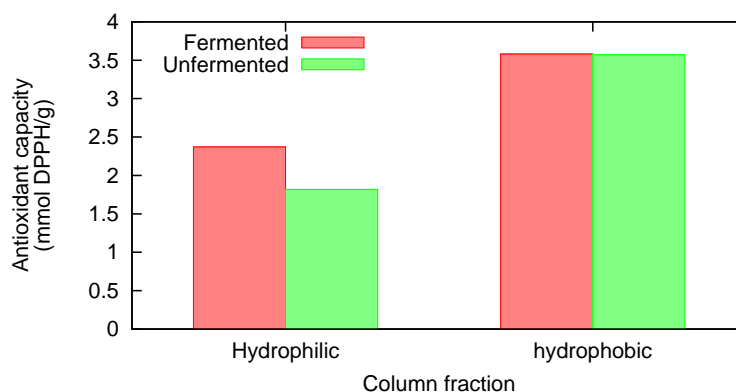
Figure 3.3: Antioxidant capacities (singlicate measurements) of preliminary HIC fractions of fermented and unfermented rooibos extracts

A second fractionation was performed on both the fermented and unfermented rooibos extracts in which five separate fractions were collected for each extract. The assay results (fig. 3.5 and fig. 3.6) show a clear increase in the antioxidant capacity of both the fermented and unfermented rooibos extracts as the hydrophobicity of the collected fractions increases. Although the total polyphenol content of the unfermented rooibos was highest in the most
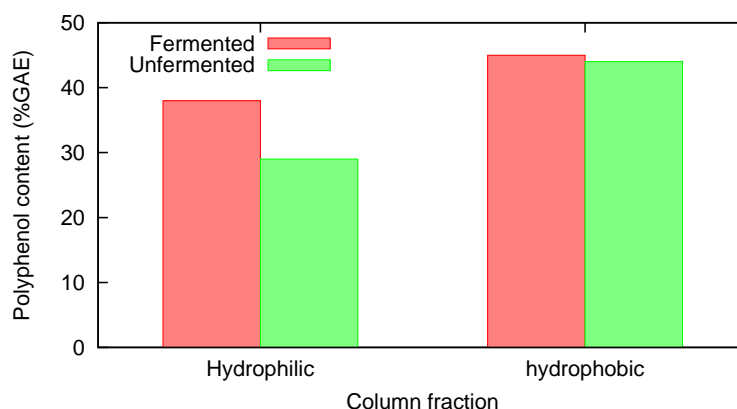
Figure 3.4: Total polyphenol concentrations (singlicate measurements) of preliminary HIC fractions of fermented and unfermented rooibos extracts

hydrophobic fractions, the most hydrophobic fractions of the fermented rooibos were not as highly enriched as the intermediate fractions. Since this was a preliminary investigation, merely aimed at ascertaining the trend of the enrichment achived with the HIC fractionation, the samples were not analysed in duplicate, it cannot be determined whether this decline is statistically significant.

The unfermented rooibos clearly showed a more efficient fractionation of polyphenols than the fermented rooibos – the difference between initial (more hydrophilic) fractions and later (more hydrophobic) fractions was more pronounced for the unfermented rooibos than for the fermented rooibos. Despite this difference, if the antioxidant capacities of the fermented and unfermented samples are plotted versus their polyphenol content (fig. 3.7), both data sets are well represented by the same linear correlation (correlation coefficient $r^2 = 0.940$ for the combined data set). The good linear fit, as well as the fact that the correlation line passes near the origin of the graph, is consistent with the research to date, as the antioxidant capacity of rooibos is believed to be primarily due to its polyphenol components. If large amounts of hydrophilic non-polyphenol antioxidants such as ascorbate had been present, neither of these properties would have held true. Interestingly, in all cases, the more hydrophilic fractions of the fermented rooibos showed higher polyphenol content and antioxidant capacity. This could be explained by an increase in polarity caused by oxidation reactions during fermentation.
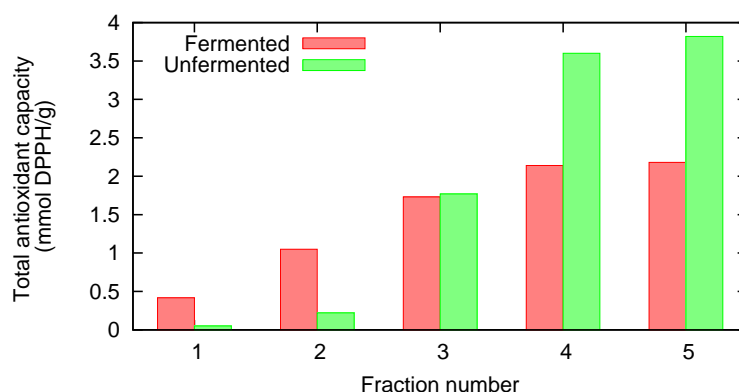
Figure 3.5: Comparison of the antioxidant capacity of HIC fractions of fermented and unfermented rooibos. All assays performed in singlicate. Fraction 1: most hydrophilic, fraction 5: most hydrophobic.
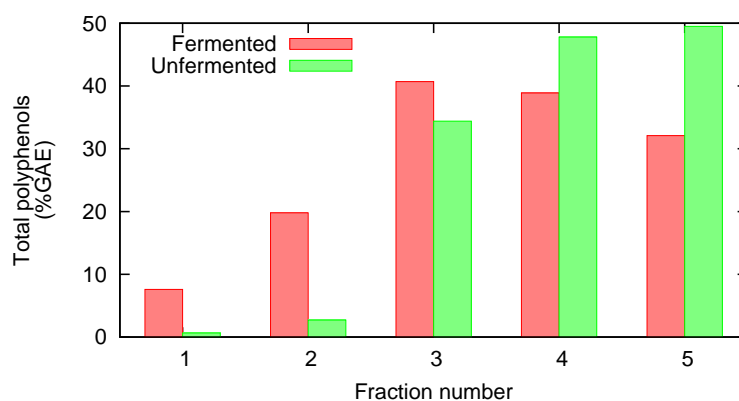

Figure 3.6: Comparison of the polyphenol concentrations of HIC fractions of fermented and unfermented rooibos. All assays performed in singlicate. Fraction 1: most hydrophilic, fraction 5: most hydrophobic.

Qualitative HPLC analysis of unfermented rooibos HIC fractions

Subsequent to the polyphenol and antioxidant analyses of the rooibos extracts, the unfermented rooibos HIC column fractions were selected for further study, as they showed a more pronounced difference between the polyphenol content of the initial and later fractions.

Chromatograms obtained by RP-HPLC (figure 3.8) were recorded at 288 nm for easy visualisation of the aspalathin and nothofagin peaks. Peaks were identified by comparison of the retention times and UV spectra with those of
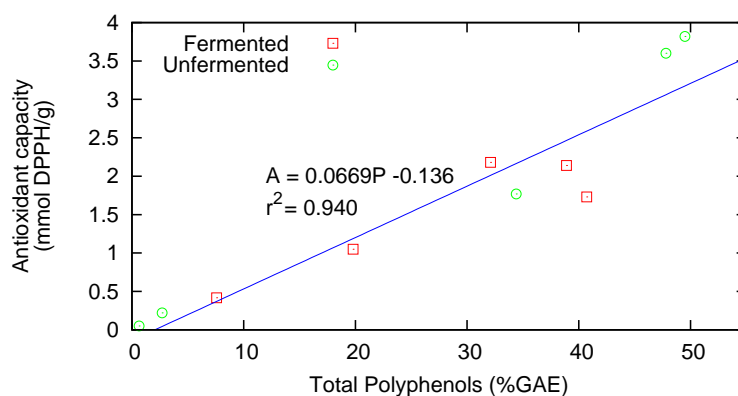
Figure 3.7: Relationship between polyphenol concentration and antioxidant capacity of HIC fractions of fermented and unfermented rooibos (see fig. 3.5 and 3.6)

pure standards. A representative hydrophobic fraction, a more hydrophilic fraction and, for comparison, a chromatogram for unfermented rooibos are shown. It is clear from figure 3.8 that the HIC fractionation system successfully produced samples of differing hydrophobicity. It is also clear, however, that the HIC fractionation did not result in completely disjunct fractions, with some components distributed to a significant extent over more than one fraction. The two major rooibos polyphenols, aspalathin and nothofagin (retention times 22.5 and 25.4 minutes respectively), however, were mostly present in the hydrophilic fraction, with only trace amounts remaining in the hydrophobic fraction. One fermented and one unfermented HIC fraction of intermediate hydrophobicity (fraction 3 in figures 3.5 and 3.6) were selected for further investigation, because they were considered to be representative fractions, the fermented and unfermented fractions having similar antioxidant capacity and polyphenol content.

Quanitative HPLC analysis of fermented and unfermented rooibos

Unfermented and fermented rooibos extracts, and HIC column fractions of intermediate hydrophobicity derived from these extracts, were subjected to HPLC-DAD analysis. A number of polyphenols, known to occur in rooibos, were used to generate standard curves in order to identify and quantify these compounds in the samples under investigation.

The results of these analyses are shown in table 3.1. Paracoumaric acid, a minor rooibos component, was not detected. The HIC fractions of the fermented and unfermented rooibos exhibited higher levels of aspalathin than
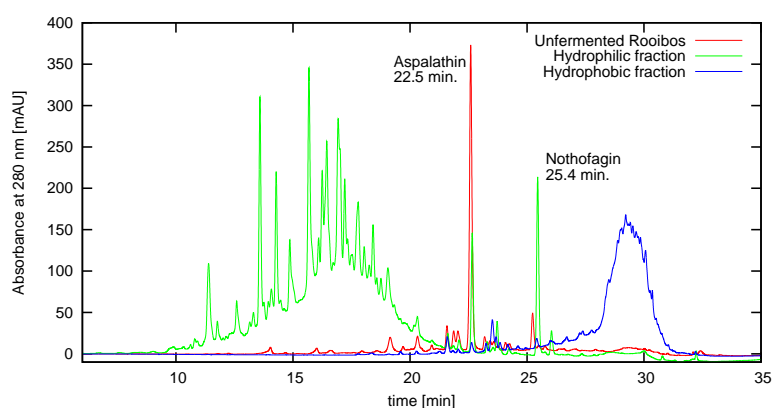
Figure 3.8: RP-HPLC of unfermented rooibos and hydrophobic and hydrophilic unfermented rooibos fractions. Zorbax SB-C18 column, particle size 3.5 μm, 3.0×150 mm; see page 21 for elution conditions.

the extracts from which they were made, which should be understood in the light of the fact that aspalathin has an intermediate hydrophobicity. Conversely, the phenolic acids (more hydrophilic) and nothofagin (slightly more hydrophobic) were less well represented in the HIC fractions. Rutin hydrate was the only flavonoid apart from aspalathin that was markedly concentrated in both HIC fractions. Although, like nothofagin, it has one fewer phenolic hydroxyl group than aspalathin, the decrease in hydrophilicity is compensated for by the disaccharide substituent at C3.

NP-HPLC analysis of aqueous rooibos extract

NP-HPLC was carried out on aqueous extracts of unfermented rooibos in an attempt to identify tannin-like compounds. A published NP-HPLC method for the analysis of proanthocyanidins [108] was used to detect and resolve similar compounds in an aqueous extract of unfermented rooibos.

Four peaks eluting at regular intervals were observed between 20 and 30 minutes. A three-dimensional representation of the data obtained with the diode-array detector is shown in figure 3.9 A. The UV absorbance spectra of these peaks were very similar – a representative spectrum is shown in figure 3.9 C – and strongly resembled the spectra of aspalathin (fig. 3.9 B) and nothofagin (not shown). These compounds all exhibit a maximum at 285 nm and a minor peak at 245 nm. The absorbance profile was markedly different from the spectra of typical flavon-3-ols such as quercetin (fig. 3.9 D), which exhibits a maximum absorbance at 256 nm under the same conditions, and

Table 3.1: Quantitative analysis of polyphenols in rooibos samples

Compound name	Unfer- mented rooibos	Unfermented HIC fraction [1]	Fermented rooibos	Fermented HIC fraction[1]
Aspalathin	10%	65%	0.53%	1.0%
Nothofagin	1.3%	0.60%	0.11%	0.06%
Isovitexin	0.18%	ND [2]	ND	0.11%
Luteolin	ND	ND	0.084%	ND
Vitexin	0.43%	1.1%	0.46%	0.42%
Quercetin-3-β-D-glycoside	0.16%	ND	0.13%	0.08%
Quercetin dihydrate	ND	ND	0.075%	ND
Rutin Hydrate	0.60%	0.94%	ND	0.59%
3,4 DHBA	ND	ND	0.22%	0.034%
Caffeic acid	0.022%	ND	0.04%	ND
Ferulic acid	ND	ND	0.17%	ND
p-Coumaric acid	ND	ND	ND	ND
Vanillic acid	0.023%	ND	0.09%	ND
Syringic acid	0.64%	ND	ND	0.11%

[1]**fraction 3 in figure 3.5**
[2]**not detected**

was also different from the UV spectrum of procyanidin B1 and procyanidin B2 ($\lambda_{max} = 280$ nm [109].)

Fractionation was carried out to isolate the HPLC fractions corresponding to the four peaks identified by HPLC-DAD (shown as shaded areas in figure 3.10). The fractions were collected and subjected to ESMS. Although the concentration of the samples was not high enough for structural elucidation, the molecular masses of the compounds in question could be determined with reasonable confidence, and are shown in table 3.2. The difference in molecular mass between fraction 1 and fractions 2 and 3 was 288 Da, and the difference between fractions 4 and fractions 2 and 3 was 272 Da. The calculated mass of a pentahydroxy dihydrochalcone aglycone (such as the aglycone of aspalathin) is 288 Da, and of a tetrahydroxy dihydrochalcone aglycone (such as the aglycone of nothofagin) is 272 Da. Typical molecular masses for singly linked proanthocyanidin oligomers are 578 Da (dimer, e.g. Procyanidin B), 866 Da (trimer) and 1154 Da (tetramer) [110]. These results are therefore consistent with the hypothesis that these compounds are tannin-like flavonoid oligomers containing dihydrochalcone monomers.
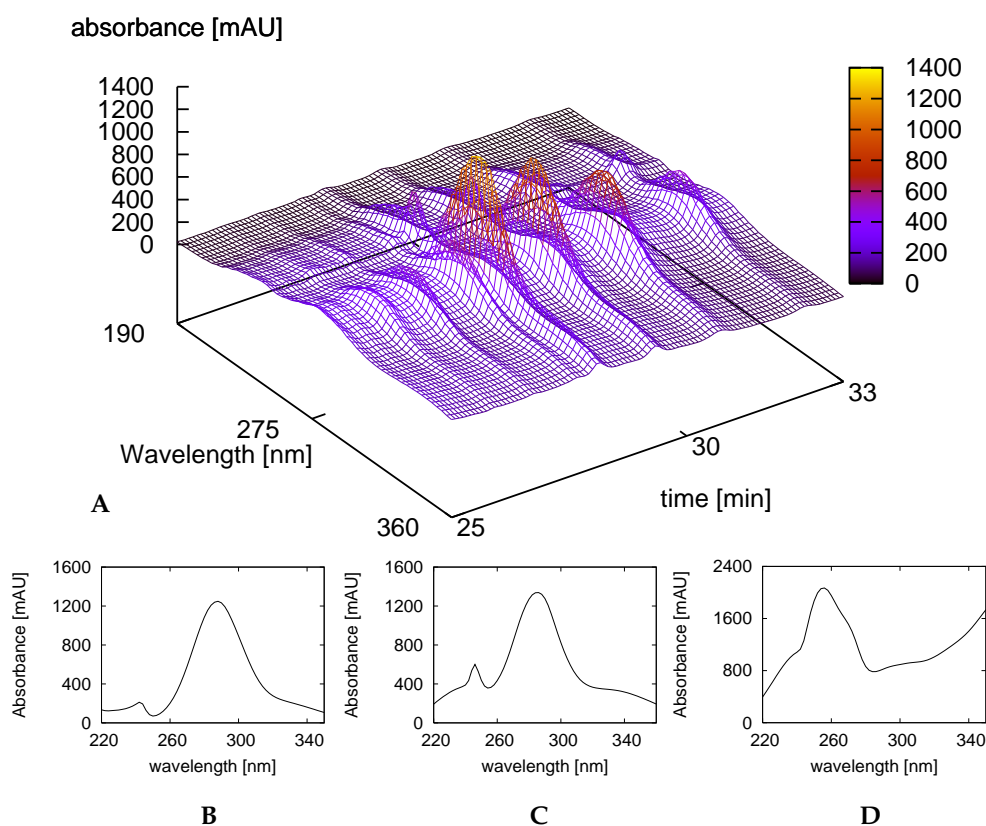
Figure 3.9: HPLC-DAD analysis of unfermented rooibos aqueous extract. NP-HPLC on Supelcosil 5μm LC-SI column, 250×4.6 mm, see p. 21 for elution conditions. **(A)** Diode-array detector peak profile over area of interest. **(B)** UV spectrum of aspalathin standard. **(C)** UV spectrum of major peak (peak 1 in fig. 3.10). **(D)** UV spectrum of Quercetin dihydrate standard.

3.4 Summary

In this chapter, it was shown that rooibos tea can be separated into fractions of different hydrophobicity by HIC. These fractions have different polyphenol contents, and their radical scavenging antioxidant capacities vary in a direct linear relationship with their polyphenol content.

As can be seen from the sample chromatograms (fig. 3.8), the marker compounds used to quantify the degree of enrichment are only illustrative of the range of compounds in rooibos. Since many peaks are still to be identified, the choice of standards was influenced by work done by Bramati *et al.* [9]. Although they used different HPLC conditions, sample material and sample
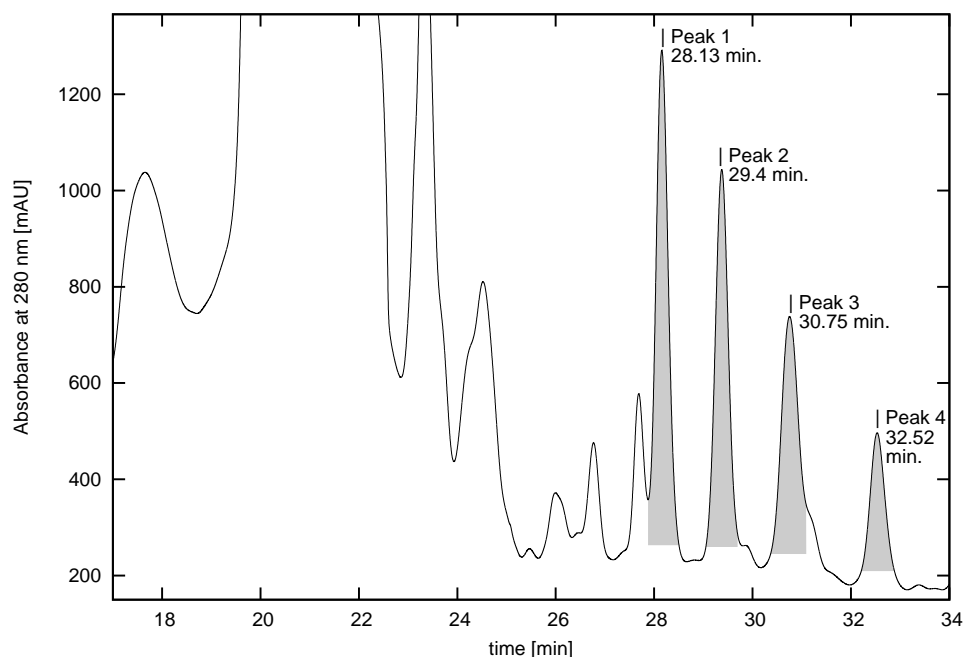
Figure 3.10: NP-HPLC fractionation of unfermented rooibos aqueous extract (Supelcosil 5μm LC-SI column, 250×4.6 mm, see p. 21 for elution conditions.) Shaded areas indicate peak collection windows for ESMS analysis.

Table 3.2: Molecular masses of compounds present in NP-HPLC fractions corresponding to peaks 1–4, fig. 3.10

Peak	Molecular Mass
1	613
2	901
3	901
4	1173

preparation methods from those employed in this study, a number of compounds in common with those identified by their group (viz. Aspalathin, Nothofagin, Isovitexin, Luteolin, Vitexin, Quercetin-3-β-D-glycoside, Quercetin dihydrate, Rutin Hydrate, 3,4 DHBA, Caffeic acid, Ferulic acid, Vanillic acid and Syringic acid) could be identified.

It was further shown that certain groups of polyphenols can be substantially enriched in rooibos by the use of HIC. This was demonstrated both by qualitative and quantitative HPLC analysis. The HIC fractions of intermediate hydrophobicity were strongly enriched with respect to their aspalathin content. Vitexin and rutin hydrate are very similar to aspalathin in hydrophobicity,

and were also enriched in the HIC fractions. Nothofagin only differs from aspalathin by the absence of one hydroxyl group, and is only slightly more hydrophobic. It was therefore also well represented in the HIC fractions, at a level approximately 60% of that in the original extract. A number of other flavonoids known to occur in rooibos were also detected. Apart from syringic acid, the least hydrophilic phenolic acid assayed, none of the phenolic acids (more hydrophilic than aspalathin) were represented in the HIC fractions. Luteolin, quercetin and quercetin glucoside each have less phenolic hydroxyl groups than nothofagin and have a closed C-ring, unlike nothofagin or aspalathin [4, 12, 76] and are consequently more hydrophobic than nothofagin. They were therefore not present in the intermediate-hydrophobicity HIC fractions in significant quantities.

Evidence is furthermore presented that indicates the possible existence of tannin-like compounds at low concentrations in unfermented rooibos. There appears to be a correlation between the molecular mass of these compounds and the molecular mass of the aglycones of the major polyphenols of rooibos, but additional experiments will need to be carried out to identify these compounds.

Although the glycation patterns of the main polyphenols present in rooibos may inhibit or prevent the formation of tannins or tannin-like compounds, their corresponding aglycones may well take part in polymerisation reactions, forming tannin-like compounds in low concentrations.

Chapter 4

Cytochrome P450 enzymes and adrenal steroidogenesis

"The air we breathe contains a most deadly poison, called by chemists azotic gas, which, by its being mixed with what is called vital air, (oxygen gas,) becomes necessary to our existence"
— The Mirror of Literature, Amusement, and Instruction: August 2, 1828.

4.1   Introduction

As discussed in chapter 2, rooibos is widely believed to be helpful in diseases that are linked to chronic stress. The hormones that orchestrate the stress response are produced by specific cytochrome P450-dependent systems under the control of the homeostatic feedback loops of the hypothalamic-pituitary-adrenal (HPA) axis [26]. The interaction of rooibos with these systems will be investigated in the following chapters, so a brief introduction to the biochemistry and physiology of P450 enzyme systems and the HPA axis will now be presented.

4.2   Cytochrome P450 enzymes

Cytochrome P450 (P450) is the collective name for a superfamily of enzymes found in all three domains of life: eukaryotes, archaea and bacteria. P450 enzymes exhibit a wide diversity and as of October 2006, 6422 P450 genes had been fully sequenced (see table 4.2) [24].

In humans, P450 enzymes are most prominent in the liver and the adrenal gland, but they also play important roles in a wide range of tissues [111], catalysing reactions involved in such diverse pathways as the synthesis of bile acids and cholesterol, the activation and degradation of Vitamin D, and the metabolism of drugs and steroids [112]. P450 enzymes are best known for their role as catalysts of a wide variety of mono-oxidations, such as hydroxylation and epoxidation, but some also take part in electron-transfer reactions, for example the reduction of epoxides and N-oxides [113].

Table 4.1: P450 genes, classified by kingdom.

Animals:	2279 in	99	families
Plants:	2311 in	94	families
Fungi:	1001 in	282	families
Bacteria:	621 in	177	families
Protists:	210 in	51	families
Archaea:	8 in	5	families
Total:	6422 in	708	families

In common with hemoglobin, myoglobin, catalases, peroxidases and cytochrome b, the P450 enzymes all contain a protoporphyrin IX ring structure with an iron atom tetracoordinated between four nitrogen atoms [114, 115]. The P450 enzymes are distinguished from other proto-heme cytochromes by the fact that the fifth coordination position[1] is occupied by a cysteine-derived sulphur atom instead of a histidine-derived nitrogen, as seen in figure 4.1. This alters the electron distribution in the heme group, allowing for the activation of molecular oxygen and resulting in the unique spectral properties of the P450 enzymes [112].

Liver P450 enzymes have broad substrate specificity and metabolise a range of xenobiotics such as toxins and drugs, usually by increasing the solubility
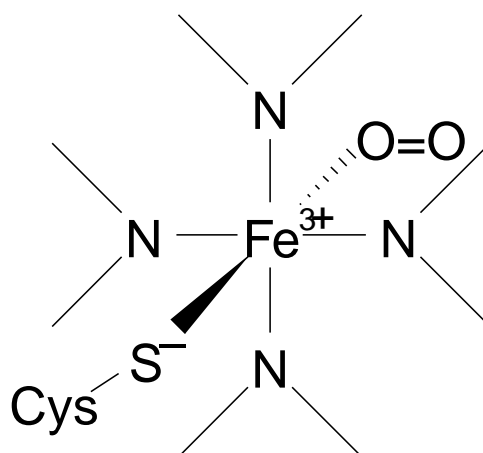
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[1]proximal to the peptide backbone

Figure 4.1: Protoporphyrin nucleus of P450, shown with bound oxygen molecule.

of hydrophobic substrates via hydroxylation and epoxidation reactions to facilitate excretion [112]. In certain cases, however, this process leads to the formation of quinones and other harmful intermediates.

The levels of liver P450 enzymes are increased in the presence of their substrates, usually due to an increase in the rate of gene transcription, but also by stabilisation of gene products [112]. This effect is often exploited to increase the levels of P450 enzymes in vivo for research purposes. The induction of P450 enzymes also has a major influence on the pharmacokinetics of drugs, and is therefore of commercial importance [112].

In the adrenal gland, a number of P450 enzymes with much narrower substrate specificity are responsible for many of the reactions in steroid hormone biosynthesis. In contrast to the liver P450 enzymes, adrenal P450 enzymes are constitutively expressed and are not induced by increased substrate concentration. This is necessary, because whereas an influx of a toxic or foreign substance must be met with increased enzyme activity, the flux through the steroidogenic pathways needs to be under homeostatic control.

With the exception of some soluble bacterial forms, all P450 enzymes are localized in cellular membranes – in the endoplasmatic reticulum (ER) and also in the inner mitochondrial membrane [111]. However, they are by no means restricted to these membranes, since they have also been isolated from the Golgi apparatus, peroxisome membranes and even the outer nuclear membrane [111].

P450 enzymes typically form part of multicomponent electron-transfer chains, which are referred to as "P450-containing systems". Two broad

classes of P450-containing systems are involved in P450 enzyme catalysed reactions – class I and class II [116].

In bacteria and in mitochondria, three proteins are involved [116]:

- the class I P450 enzyme
- adrenodoxin (in mitochondria) and ferredoxin (in bacteria)
- the flavoproteins NADPH-adrenodoxin reductase (in mitochondria) and NADH-ferredoxin reductase (in bacteria)

Adrenodoxin and ferredoxin are Fe_2S_2 iron-sulphur proteins that reduce the P450 enzyme [116]. These iron-sulphur proteins are in turn reduced by adrenodoxin reductase or ferredoxin reductase [116], which receive reducing equivalents from NADH or NADPH, respectively:

$$NAD(P)H \longrightarrow FAD\text{-protein} \longrightarrow Iron\text{-Sulphur protein} \longrightarrow P450$$
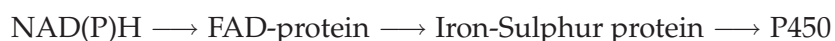
In microsomal systems, associated with the ER of eukaryotic cells, only two proteins are involved [116]:

- the class II P450 enzyme
- NADPH-cytochrome P450 reductase

The P450 enzyme receives reducing equivalents from P450 reductase, an FAD and FMN-dependent enzyme, which receives reducing equivalents from NADPH [116].

$$NAD(P)H \longrightarrow FAD\text{-FMN-protein} \longrightarrow P450$$

For both of these systems, the net P450-catalysed mono-oxygenase reaction is the same [116]:

$$RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O$$
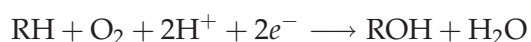
In the process of this reaction, the enzyme goes through a cycle of reactions. To illustrate this cycle, a hypothetical hydroxylation reaction R $\longrightarrow$ R-OH, represented schematically in figure 4.2 will be discussed. Starting with the heme iron in the oxidised state without bound ligand:
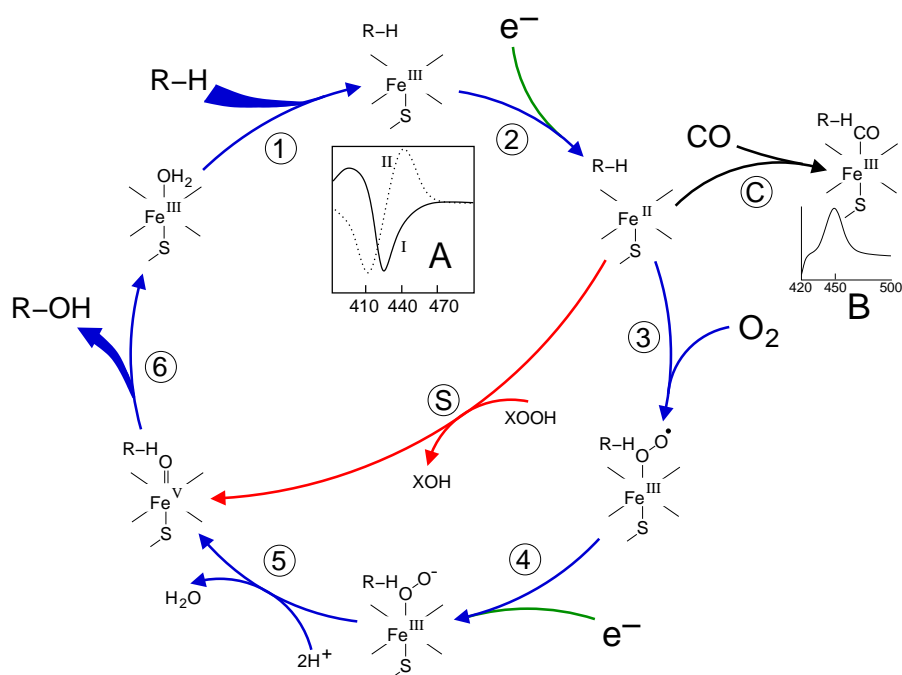
Figure 4.2: The P450 catalytic cycle, shown for a hypothetical hydroxylation of substrate "R" – R ⟶ R-OH. **A**: Type I and type II substrate binding spectra; **B**: CO-induced P450 spectrum. Refer to text for nomenclature and discussion.

1 The substrate binds to the active site of the enzyme, in close proximity to the heme group, on the side opposite to the peptide chain. The bound substrate induces a change in the conformation of the active site, displacing a water molecule from the distal axial coordination position of the heme iron [116] changing the state of the heme iron from low-spin to high-spin [117]. This gives rise to a change in the spectral properties of the enzyme, with an increase in absorbance at 390 nm and a decrease at 420 nm. This can be measured by difference spectrometry and is referred to as the "type I" difference spectrum (see inset graph in fig. 4.2). Some substrates cause an opposite change in spectral properties, a "reverse type I" spectrum, by processes that are as yet unclear. Inhibitors and certain substrates that bind directly to the heme iron give rise to the type II difference spectrum, with a maximum at 430 nm and a minimum at 390 nm (see inset graph in fig. 4.2). If no reducing equivalents are available, this complex remains stable, allowing the degree of binding to be determined from absorbance measurements *in vitro* [23].

2 The change in the electronic state of the active site favours the transfer of an electron from NAD(P)H [118]. This takes place via the electron transfer chain, as described above, reducing the ferric heme iron to the ferrous state.

3 Molecular oxygen binds covalently to the distal axial coordination position of the heme iron. The cysteine ligand is a better electron donor than histidine, with the oxygen consequently being activated to a greater extent than in other heme proteins. However, this sometimes allows the bond to dissociate, the so-called "decoupling reaction", releasing a reactive superoxide radical, interrupting the catalytic cycle [116].

4 A second electron is transferred via the electron-transport system, reducing the dioxygen adduct to a negatively charged peroxo group. This is a short-lived intermediate state.

5 The peroxo group formed in step 4 is rapidly protonated twice by local transfer from surrounding amino-acid side chains, releasing one mole of water, and forming a highly reactive iron(V)-oxo species [116].

6 Depending on the substrate and enzyme involved, P450 enzymes can catalyse any of a wide variety of reactions. A hypothetical hydroxylation is shown in this illustration. After the product has been released from the active site, the enzyme returns to its original state, with a water molecule returning to occupy the distal coordination position of the iron nucleus.

S An alternative route for mono-oxygenation is via the "peroxide shunt": interaction with single-oxygen donors such as peroxides and hypochlorites can lead directly to the formation of the iron-oxo intermediate, allowing the catalytic cycle to be completed without going through steps 3, 4 and 5 [23]. A hypothetical peroxide "XOOH" is shown in the diagram.

C If carbon monoxide (CO) binds to reduced P450, the catalytic cycle is interrupted. This reaction yields the classic CO difference spectrum with a maximum at 450 nm.

The spectral properties of P450 enzymes were not only instrumental to their discovery, but are also useful in their investigation. The CO difference spectrum can be exploited to determine the concentration of cytochrome P450 in a solution or suspension. The difference spectra evoked by the binding of substrates and inhibitors can be used to determine degrees of binding and inhibition directly, without the need for chemical assays. These methods will be discussed in more detail in chapter 5.

4.3 Adrenal steroidogenesis

The steroid hormones secreted by the adrenal gland are produced by a specific group of P450 enzymes and a hydroxysteroid dehydrogenase. Unlike many other hormones which are produced in advance and released in response to physiological demand, adrenal steroid hormones enter the bloodstream immediately upon production, with the rate of synthesis being modulated in response to physiological events [26] by the hormones of the HPA axis.

The first step in adrenal steroidogenesis is the cleavage of the side chain of cholesterol by three consecutive hydroxylation reactions, catalysed by cytochrome P450 side chain cleavage (CYP11A1), yielding pregnenolone (P5) and isocapraldehyde as products [119]. P5 is converted to progesterone (P4) by 3β-hydroxysteroid dehydrogenase (3β-HSD). P5 and P4 stand at a branch point in adrenal steroidogenesis, as they are both acted upon by cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17). As shown in figure 4.3, the 17α-hydroxylation of P5 and P4 and the subsequent cleavage of the side chain from the intermediates 17-hydroxypregnenolone (17OH-P5) and 17-hydroxyprogesterone (17OH-P4) yield the androgen precursors dehydroepiandrosterone (DHEA) and androstenedione (A4), respectively, although the

production of DHEA from 17OH-P5 is negligible in *ex vivo* ovine adrenal preparations [120].

3βHSD catalyses the conversion of 17OH-P5 and DHEA to 17OH-P4 and A4, respectively. Cytochrome P450 steroid 21-hydroxylase (CYP21) catalyses the hydroxylation of P4 and 17OH-P4 to deoxycorticosterone (DOC) and deoxycortisol (S) respectively. These intermediates are subsequently hydroxylated by cytochrome P450 steroid 11β-hydroxylase (CYP11B1), yielding the adrenal glucocorticoids corticosterone (B) and cortisol (F). Aldosterone synthase (CYP11B2) catalyses the conversion of DOC to the mineralocorticoid aldosterone.

The relative activities of CYP17 and CYP21 are crucial to the control of adrenal steroidogenesis. A number of mutations that affect the activity of CYP17 and therefore cause serious hormonal dysfunction, resulting in conditions such as congenital adrenal hyperplasia (CAH) [121, 122], have been identified. It has also been shown that compounds isolated from the shrub *Salsola tuberculatiformis botch*, which causes prolonged gestation in karakul sheep, inhibit CYP17 and CYP21 (personal communication, A.C. Swart), as well as CYP11B1, which catalyses the final step in cortisol biosynthesis [123].

Adrenal glucocorticoids participate in the control of whole body homeostasis, regulating physiological processes. Chronic physical or psychological stress leads to elevated basal glucocorticoid plasma levels which in turn leads to physiological disturbances at the endocrine level [124]. Glucocorticoid levels are regulated by the HPA axis.

4.4 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal axis or HPA is a term which refers to the interaction between the hypothalamus, the pituitary and the adrenal glands, which is manifested as a set of homeostatic feedback loops.

The hypothalamus is a conical region of the brain located directly below the thalamus, forming the major part of the ventral and medial regions of the diencephalon (fig. 4.4). It is connected by neuronal pathways to various parts of the brain, and exerts control over the endocrine system by its effects on the pituitary gland. Neuropeptides are released into the posterior pituitary, whence they are released into the bloodstream. The hypothalamus also secretes releasing and inhibitory hormones into the hypothalamo-hypophysial portal vessels, controlling the secretion of the hormones of the anterior pituitary.
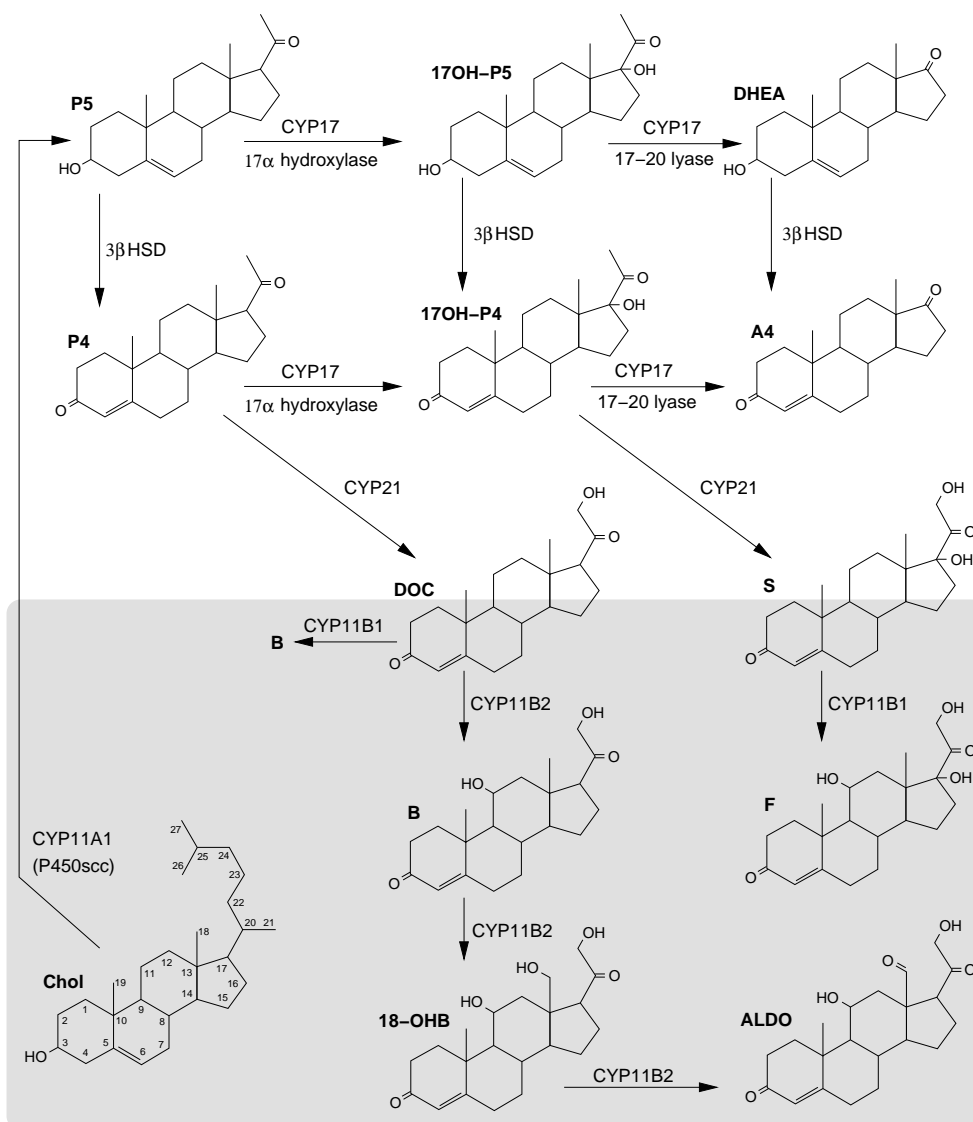
Figure 4.3: Adrenal steroidogenic reactions. The arrows that originate within the shaded area represent mitochondrial reactions; all other reactions take place on the endoplasmatic reticulum. Abbreviations: **Chol**: cholesterol; **P5**: pregnenolone; **17OH-P5**: 17-hydroxypregnenolone; **DHEA**: dehydroepiandrosterone; **P4**: progesterone; **17OH-P4**: 17-hydroxyprogesterone; **A4**: androstenedione; **DOC**: 11-deoxycorticosterone; **S**: 11-deoxycortisol; **B**: corticosterone; **F**: cortisol; **18-OHB**: 18-hydroxycorticosterone; **ALDO**: aldosterone; **CYP11A1**: cytochrome P450 cholesterol side chain cleavage; **CYP17**: cytochrome P450 17α-hydroxylase/17,20 lyase; **3βHSD**: 3β-hydroxysteroid dehydrogenase; **CYP21**: cytochrome P450 steroid 21-hydroxylase; **CYP11B1**: cytochrome P450 11β-hydroxylase; **CYP11B2**: aldosterone synthase
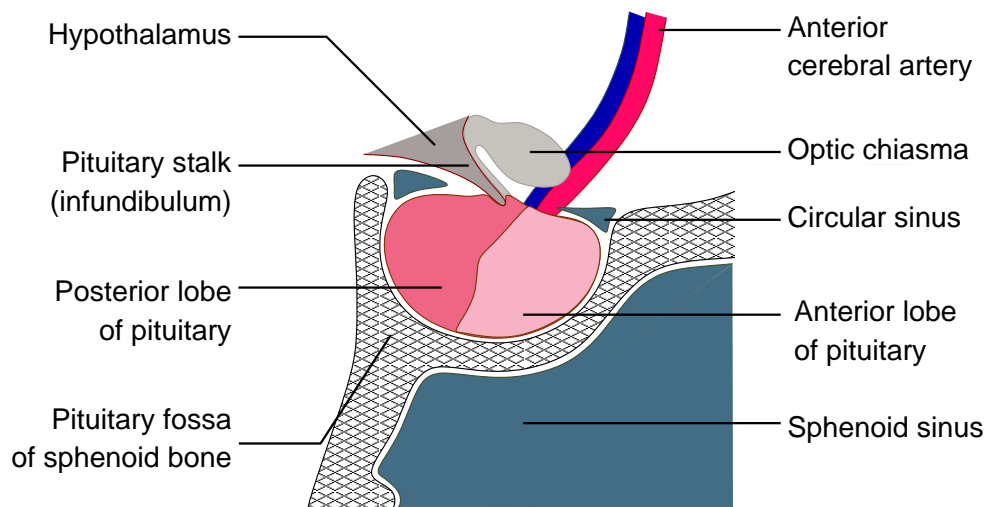
Figure 4.4: The pituitary gland and hypothalamus, sagittal section showing location relative to the optic chiasma and sphenoid bone. Re-drawn from Gray's Anatomy of the Human Body, 20th US ed.

Certain pituitary hormones direct a range of metabolic processes via their effects on target organs – in the case of the somatotrope human growth hormone (hGH), the lactotrope prolactin (PRL) and the gonadotropes luteinising hormone (LH) and follicle stimulating hormone (FSH). Others exert their influence via their stimulating effects on other endocrine glands – in the case of the thyrotrope thyroid stimulating hormone (TSH) and the corticotrope adrenocorticotropin (ACTH) [113].

The HPA axis comprises the following hormonal actions as illustrated in figure 4.5 [26, 27]:

1   corticotropin releasing hormone (CRH), also called corticotropin releasing factor (CRF), is secreted by the hypothalamus

2   CRH is carried by the hypothalamo-pituitary portal vessels to the anterior pituitary

3   CRH stimulates the anterior pituitary to release ACTH

4   ACTH stimulates the secretion of the glucocorticoid hormones corticosterone and cortisol by the adrenal gland

5   The glucocorticoids are transported by the circulatory system to the various organs where they exert their effects.

6   the glucocorticoids exert inhibitory effects on the hypothalamus and
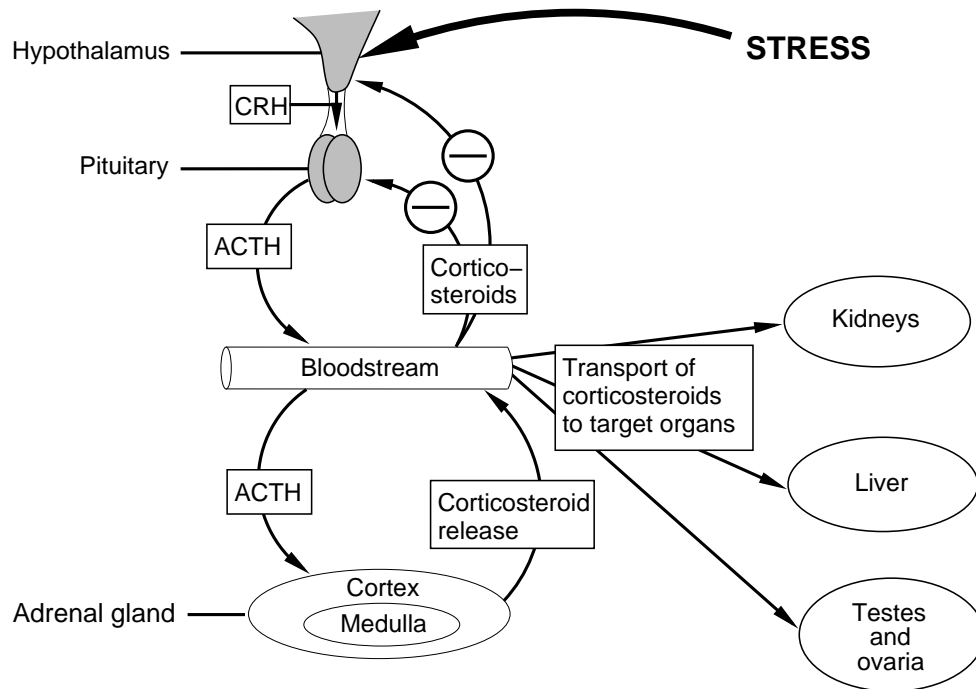   pituitary, thereby creating a negative feedback system (see figure 4.5).

Figure 4.5: The HPA axis: secretion and transport of hormones to target organs, and
negative feedback due to effects of corticosteroids on hypothalamus and pituitary.

In general, activation of the HPA axis is an adaptive, transient response both
to normal environmental stimuli and to stressful stimuli. Normal stimuli
include the light/dark cycle, physical exertion and normal temperature vari-
ations. Stressful stimuli include physical stress such as extreme tempera-
ture variation, prolonged fasting and physical injury [26], as well as emo-
tional stress such as bereavement [125] and inter-individual conflict [126],
especially in early life [127, 128]. Such stressors, usually categorised as "dis-
tress", can be contrasted with the positive stress, "eustress", associated with
overcoming challenges [129], performing physical exercise [130] and mirth-
ful laughter [131], which can all have positive effects on immunity and sub-
sequent reactions to negative stress.

Chronic physical or psychological stress and the concomitant chronic activa-
tion of the HPA axis leads to chronically raised CRH levels in the cerebro-
spinal fluid. This condition can have different effects on the HPA axis, result-
ing in extended elevation or depression of glucocorticoid levels depending

on the timing and the nature of stressors [132–134]. Chronic activation of the HPA axis is an undesirable situation, which must be managed by medication or by such techniques as cognitive behavioural stress management [135]. Chronic elevation of glucocorticoids results in the inhibition of reproduction and growth, the inhibition or inappropriate activation [136] of the inflammatory reaction and the inhibition of all the major components of the immune response, as well as the promotion of visceral adiposity, insulin resistance, hypertension and "low turnover" osteoporosis. Elevated glucocorticoid levels have also been associated with depression, chronic anxiety, fatigue and sleep disturbances [137].

In the light of the central role of adrenal P450 enzymes in the stress response, it is important to investigate the interaction of these enzymes with rooibos in order to more completely investigate the stress-relieving properties of rooibos. This line of investigation will be further developed in chapter 5.

Chapter 5

Influence of *Aspalathus linearis* extracts on substrate binding to adrenal steroidogenic P450 enzymes

"The so-called enzyme action, ascribed by physiology to the various digestive juices, is in reality the product of an activity of the lower part of man's astral organization"
— *Man or Matter* (Ernst Lehrs)

5.1 Introduction

Rooibos is believed to be beneficial in conditions related to chronic stress as has been discussed in chapter 2. Although the antioxidant nature of rooibos is firmly established, it cannot be directly linked to the beneficial effects of rooibos on clinical conditions related to stress. The stress response, however, is mediated to a large extent by the adrenal corticosteroids, corticosterone (B, fig. 4.3) and cortisol (F, fig. 4.3), which are synthesised by P450-containing systems. In this study the influence of rooibos on CYP17 and CYP21 was investigated, since CYP17, standing as it does at the branch point of gluco-corticoid and androgen biosynthesis, plays a pivotal role in adrenal steroido-genesis. As discussed previously, CYP17 metabolises P5 to 17OH-P5 and subsequently to DHEA [26]. P4 is also metabolised to 17OH-P4 and, in some

species, to A4 [112]. However, while CYP17 may channel precursors into the androgen pathway, CYP21 channels these steroids into the glucocorticoid pathway, converting P4 to deoxycorticosterone (DOC, fig. 4.3) and 17OH-P4 to deoxycortisol (S, fig. 4.3) [112]. DOC and S are subsequently converted by CYP11B1 to B and F, respectively [112]. Compounds that affect the binding of native steroid substrates to their enzymes may inhibit these reactions. It is possible that inhibition of CYP17 or CYP21 may influence the outcome of steroid metabolism, with concomitant effects on the physiological stress response.

The tissue preparations used in the present study contain P450 enzymes, including ovine CYP21 and CYP17, both of which are capable of utilising progesterone (P4) and 17OH-P4 as substrate. Swart *et al* [120] have shown that although CYP17 expressed in the ovine adrenal is in principle capable of converting P5 to DHEA via 17OH-P5, its lyase activity is negligible in adrenal preparations. The major metabolite of P4 metabolism was also found to be 17OH-P4 in adrenal microsomes. Despite the lack of lyase activity in microsomal preparations, CYP17 exhibits a typical type I substrate-induced difference spectrum upon binding to 17OH-P5 [120]. Although 17OH-P4 also elicits a type I spectrum, this may be due to the binding of CYP21 to the steroid metabolite. Interestingly, in heterologous expression systems it was shown that ovine CYP17 catalysed the conversion of P5 and P4 to their respective androgens [120]. These findings underline the complexity of these enzymes in adrenal steroidogenesis.

Enzymes are functional proteins that catalyze biological reactions. The catalytic activity of an enzyme is localized in a specific region called the catalytic site, and may or may not be dependent on a prosthetic group. The three-dimensional structure, the charge distribution and the distribution of hydrophilic and hydrophobic domains surrounding the active site are all crucial to the binding of the substrate. In the case of large, complex enzymes, like most of the members of the P450 family, there are structural elements which are specifically involved in the entry of reactants and the exit of products to and from the catalytic region, and others that facilitate the binding of redox partners [138]. The three-dimensional structure of the enzyme is therefore crucial to the activity and specificity of the enzyme.

A wide range of molecules can inhibit enzyme activity, acting in different ways. A distinction is made between reversible and irreversible inhibition, with only those molecules that covalently modify the enzyme being classified as irreversible inhibitors [139]. Inhibitors with strong non-covalent interactions are often treated as irreversible to simplify analysis, but methods exist for the analysis of tight-binding reversible enzyme inhibitors [140].

When the substrate concentration is much lower than the enzyme concentration, and in the absence of allosteric effects, enzymes display so-called Michaelis-Menten kinetics. The Michaelis-Menten equation is derived from the analysis of a single-substrate, single-product enzyme-catalysed reaction, with the following reaction scheme [139]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$
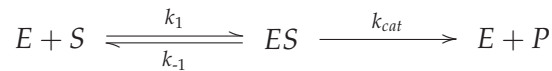
Where:

E is the enzyme;
S is the substrate;
P is the product;
k_1 is the substrate binding rate constant;
k_{-1} is the substrate dissociation rate constant; and
k_{cat} is the catalytic reaction rate constant.

Writing $\frac{k_{-1}+k_{cat}}{k_1}$ as K_M (the Michaelis constant), and recognizing that the reaction rate under conditions of substrate saturation (V_{max}) is equal to the total enzyme concentration (E_t) multiplied by k_{cat}, one can derive the familiar Michaelis-Menten equation:

$$V = \frac{V_{max}[S]}{K_M + [S]} \tag{5.1}$$

An enzyme-catalysed reaction consists of binding of substrates, reaction, and release of products. An enzyme inhibitor can have different effects on the kinetics of the enzymatic system, depending on which of these steps it interferes with:

Competitive inhibition occurs when an inhibitor binds to the same site on the enzyme as a substrate, completely preventing substrate binding. This manifests as a decrease in the apparent affinity of the enzyme for the substrate ($K_M \uparrow$), but no change in V_{max} [139].

Uncompetitive inhibition occurs in the rare case when an inhibitor binds only to the enzyme-substrate complex, completely preventing the enzymatic reaction. This usually only occurs in reactions which have multiple substrates or products [141]. By reducing the effective concentration of substrate-bound enzyme, it increases the apparent affinity of the substrate for the enzyme ($K_M \downarrow$), and reduces the apparent maximum reaction rate ($V_{max} \downarrow$). It can, however, be shown that the ratio K_M/V_{max} remains unchanged [141].

Noncompetitive inhibition occurs when an inhibitor does not interfere with substrate binding, but binds with equal affinity to the unbound enzyme and the enzyme-substrate complex, partially or completely preventing the enzymatic reaction. This type of inhibitor has no effect on K_M, but reduces the apparent maximum reaction rate ($V_{max} \downarrow$) [139].

Mixed inhibition is a term which refers to the situation which often occurs in practice, where an inhibitor exhibits a combination of more than one of the above effects, for example an inhibitor which binds with different affinities to the unbound enzyme and the enzyme-substrate complex, partly reducing the affinity of the enzyme for the substrate and partly reducing the maximum reaction rate [139].

Irreversible inhibition occurs when an inhibitor binds covalently to an enzyme, partially or completely preventing substrate binding or reaction [139].

Due to the spectral properties of P450 enzymes, as discussed previously, it is possible to investigate the binding of substrate to the active site by spectrophotometric methods, a very convenient assay which is not confounded by non-specific binding and does not require assay of steroid substrates and products. Furthermore, if reducing equivalents are not available after substrate binding, the reaction cycle cannot proceed any further, and the substrate binds and dissociates according to its adsorption equilibrium. The degree of binding can then be measured at various substrate and inhibitor concentrations, permitting calculation of the degree of inhibition. Although this system cannot detect any influence on the enzymatic reaction rate constant (k_{cat}), in the present study, equation 5.1 will be applied in modified form by using the degree of binding as a proxy for the reaction rate:

$$\Delta A = \frac{B_{max}[S]}{K_s + [S]} \tag{5.2}$$

Where:
   ΔA is the amplitude of the binding spectrum;
  B_{max} is the maximum substrate binding capacity of the enzyme;
   K_s is the substrate dissociation constant;

The data supplied by substrate binding inhibition studies will be analysed as a combination of competitive and non-competitive inhibition. The case of reversible competitive inhibition is described by the following reaction scheme [139]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

$$+$$

$$I$$

$$k_I \Updownarrow k_{-I}$$

$$EI$$
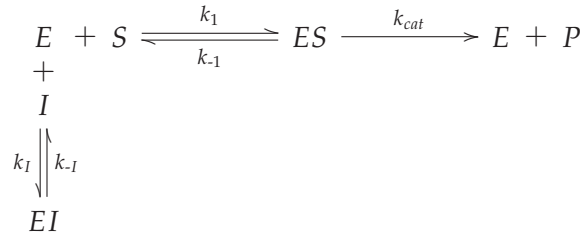
Where:

   I is the inhibitor;

   k_I is the competitive inhibitor binding rate constant; and

   k_{-I} is the competitive inhibitor dissociation rate constant;

Under steady-state conditions, this reaction scheme leads to the following re-lationship between the reaction rate and the enzyme, substrate and inhibitor concentrations:

$$V = \frac{V_{max}[S]}{\left(1 + \frac{[I]}{K_{ic}}\right)K_M + [S]} \tag{5.3}$$

Where K_{ic} is the competitive inhibition constant $\left(\frac{k_{-I}}{k_I}\right)$.

In the case where the reaction cannot take place, and only binding inhibition is under investigation, the following equilibrium is established:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$+$$

$$I$$

$$k_I \Updownarrow k_{-I}$$

$$EI$$
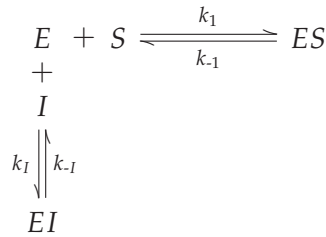
leading to a similar relationship:

$$\Delta A = \frac{B_{max}[S]}{\left(1 + \frac{[I]}{K_{si}}\right)K_s + [S]} \tag{5.4}$$

Where K_{si} is the competitive binding inhibition constant $\left(\frac{k_{-I}}{k_I}\right)$ [142].

In the light of this relationship, an apparent substrate binding constant can be defined as follows:

$$K_s^{app} = \left(1 + \frac{[I]}{K_{si}}\right) K_s \tag{5.5}$$

By analogy with uncompetitive inhibition, we can also define an apparent maximum binding capacity:

$$B_{max}^{app} = \frac{B_{max}}{1 + \frac{[I]}{K_i}} \tag{5.6}$$

Any effects on the maximum binding capacity can then be correlated by K_i, and any effects on the half-maximal binding capacity by K_{si}, yielding the following equation:

$$\Delta A = \frac{B_{max}^{app}[S]}{K_s^{app} + [S]} \tag{5.7}$$

The influence of rooibos extracts on substrate binding to adrenal cortical P450 enzymes was investigated to determine whether some of the stress-modulating effects of rooibos could be explained by effects on P450-mediated steroidogenesis. Aqueous, methanol and chloroform rooibos extracts were prepared in order to investigate their bioactivity with respect to ovine adrenal microsomal preparations containing CYP17 and CYP21. The binding of P4 and 17OH-P4 to the P450 enzymes was investigated spectrophotometrically in the presence of varying concentrations of the extracts. Bioactive rooibos fractions with differing chemical compositions, which had been separated on the basis of hydrophobicity, and which had been shown to exhibit different antioxidant capacities, were also included in this study.

Considering that mixed inhibition often occurs in practice, the analysis of reversible mixed inhibition may be simplified by assuming a combination of competitive and uncompetitive inhibition, where the inhibitor can either bind to the enzyme with or without bound substrate, resulting in no reaction taking place from either of these states without the inhibitor first dissociating from the enzyme. As only spectral assays were carried out, the data can only be analysed using the applicable parameters. Once the analysis has been completed, however, it may be possible to identify mixed inhibition.

5.2 Materials and methods

Materials and equipment

Finely ground unfermented rooibos and tea bag cut[1] fermented rooibos were provided by Benedict Technology Holdings (Pty) Ltd., Stellenbosch, South Africa. Protein assays were performed using the Pierce micro BCA protein assay reagent kit (Pierce, Rockford Ill.) with bovine serum albumin (BSA) as a standard. All reagents and standards were purchased from Merck (Darmstadt, Germany).

Difference spectra were recorded on a Cary 100 double-beam UV/Visible spectrophotometer.

Fourier analysis was performed with the Octave analysis package [144], using the FFTW library [145]. Curve fitting was done using an implementation of the nonlinear least-squares Marquardt-Levenberg algorithm in the Gnuplot graphing package [146].

Preparation of rooibos extracts

Unfermented rooibos plant material, 25 g, was placed in a glass Soxhlet extractor fitted with a double-wall condenser and a drying tube packed with granular calcium chloride. The extractor was fitted to a round-bottom flask containing 250 mL chloroform. The plant material was extracted for approximately 8 hours, until the effluent from the Soxhlet apparatus was clear. The extract was dried at reduced pressure in a rotating evaporator, and the dried extract was redissolved in 30 mL propylene glycol. The remaining plant material was subsequently extracted with 250 mL methanol in the same manner, and the dried extract was redissolved in 50 mL analytical quality water filtered through a Milli-Q™ water purification system.

Fermented rooibos, 25 g, was subjected to chloroform and methanol extraction as described above. The dried chloroform extract was redissolved in 25 mL propylene glycol, and the dried methanol extract was redissolved in 25 mL analytical quality water.

Lyophilized column fractions of rooibos had previously been prepared (see chapter 3). Fractions 1 and 5 (see fig. 3.6) were redissolved in analytical quality water at concentrations of 11.59 g/L and 16.58 g/L, respectively.

All extracts were centrifuged with a low-speed bench-top centrifuge prior to use, to separate any undissolved matter.

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[1]"Tea bag cut" is a term used for a grade of herb that has been chopped to an appropriate size for use in tea bags. Different specifications exist, but the bulk of the material might typically be between US standard mesh sizes 18 and 28 [143].

Preparation of ovine adrenal microsomes

Adrenal glands were resected from freshly slaughtered sheep and transported on ice to a $4\,°C$ cold room for further processing. The adrenal glands were decapsulated and rinsed with 1.15% KCl solution, before being homogenized in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.25 M sucrose. The homogenate was centrifuged for 20 min at $1\,000 \times g$. The supernatant fraction was centrifuged for 15 min at $12\,000 \times g$. Polyethylene glycol 800 (PEG), 50% (m/v) was added to the supernatant (final concentration, 8.5%). The mixture was stirred for 10 min at $4\,°C$ and centrifuged for 20 min at $13\,000 \times g$. The pellet was resuspended and homogenised in 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl and 1 mM EDTA. PEG was added to the suspension to a final concentration of 8.5%. The mixture was stirred and subsequently centrifuged for 20 min at $13\,000 \times g$. This procedure was repeated twice to yield a clear supernatant. The final microsomal pellet was re-suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.25 M sucrose, aliquoted and stored at $-80\,°C$ [147].

Determination of P450 concentration

The P450 concentration of the microsomal suspension was determined according to the method of Omura and Sato [148] by diluting the microsomal preparation 1:1 in 0.1 M phosphate buffer, pH 7.4, containing 10% ethylene glycol. The preparation was saturated with CO and divided into two optically matched quartz cuvettes with a path length of 1 cm. A baseline was recorded between 400 and 500 nm. Sodium dithionite (1-2 mg) was added to the sample cuvette and a spectrum was recorded. The spectrum was monitored for 10 min after addition of sodium dithionite until it was completely developed. Sodium dithionite was repeatedly added until no further increase in the absorbance at 450 nm was observed.

The amplitude of the recorded absorbance value at 450 nm was measured relative to the baseline value at 450 nm. This value was used to calculate the concentration of P450 in the preparation, according to the Beer-Lambert law:

$$\Delta A = \varepsilon c l \text{ or } c = \frac{\Delta A}{\varepsilon l}$$

Where:
A is the measured absorbance,
ε is the extinction coefficient, in this case taken to be 91 $\left[\frac{\text{L}}{\text{mmol}\cdot\text{cm}}\right]$ as reported by Omura and Sato for the heme group of P450 enzymes [148],
c is the concentration of the absorbing species, in mmol, and
l is the optical path length, in cm.

Substrate binding assays

Substrate binding assays were performed using a modified version of the method described by Kumaki *et al.* [25]. Microsomal preparations were diluted to a final P450 concentration of 0.3 μM with 0.1 M phosphate buffer at pH 7.4, containing 10% ethylene glycol. The diluted microsomes were divided into two optically matched cuvettes, and a baseline was recorded between 360 and 500 nm with a double-beam spectrophotometer. Substrate, 17OH-P4 or P4, was added to the sample cuvette, and the diluent, ethanol, to the reference cuvette. A substrate-induced difference spectrum was subsequently recorded between between 360 and 500 nm. The resulting type I difference spectrum exhibited a maximum at 390 nm and a minimum at 420 nm. After the spectrum was smoothed to remove high-frequency noise using Fourier analysis, the difference in absorbance between the maximum and minimum values was taken as a measure of the degree of substrate binding.

Saturation substrate binding assays

Substrate binding assays were carried out at final substrate concentrations of 0.8, 1.6, 3.2 and 6.4 μM. The amplitude of the type I difference spectrum was determined at each concentration, and equation 5.7 (a two-parameter hyperbolic equation) was fitted to the substrate binding data.

Binding inhibition assays

The P450 enzyme content of the adrenal microsomal fractions was determined by carbon monoxide induced difference spectra, using a millimolar extinction coefficient of 91 $\frac{L}{mmol \cdot cm}$ as reported by Omura and Sato (1964) [148]. The carbon monoxide induced difference spectrum is shown in Figure 5.1. The intensity of the absorbance at 450nm increases over time due to the slow reduction of the heme group by sodium dithionite. The spectrum exhibits a minimum around 410 nm and a maximum around 450 nm. The P450 enzyme content of the adrenal microsomal fraction containing CYP17 and CYP21 was 0.155 nmol P450/mg protein. No cytochrome P420, the inactive form of the enzyme which exhibits a peak at 420 nm, was observed.

Substrate binding assays were carried out as described above, using a substrate concentration range of 0.8–6.4 μM in the presence of varying concentrations of rooibos extract, or in the presence of varying concentrations of reconstituted rooibos LC fractions. The parameters of the fitted binding curves were recorded for calculation of inhibition parameters.

Data analysis

Spectral data was analysed by nonlinear regression to determine K_s^{app} and B_{max}^{app}. Equation 5.7 was fitted to the saturation binding curves using an implementation of the Marquardt-Levenberg nonlinear least-squares algorithm in the Gnuplot analysis package [146]. The asymptotic standard errors of the calculated parameters (B_{max}^{app} and K_s^{app}) were used as a measure of goodness of fit in further calculations.

Changes in K_s^{app} and B_{max}^{app} due to increasing inhibitor concentration were analysed by fitting the inhibition data to equations 5.5 and 5.6. A non-linear least-squares fit was done, using the standard errors calculated from the previous fits as weighting parameters. The determination of inhibition parameters was done by fitting the inhibition data to equations 5.6 and 5.5 to determine the values of K_{si} and K_i. The values are reported along with the asymptotic standard errors of the fit.

To visualize the inhibition data, the fitted inhibition parameters were substituted into equation 5.7 along with the concentrations of extract used ($[I]$ in equations 5.6 and 5.5). Equation 5.7 was then plotted together with the substrate binding data on double-reciprocal plots, a convenient linearisation of Michaelis-Menten data.

5.3 Results and discussion

Determination of P450 concentration

The P450 concentration of the microsomal preparation was determined as 1.03 μM, and the protein concentration as 6.645 mg/mL.

Difference spectra

On addition of P4 and 17OH-P4, type I substrate-induced difference spectra were observed (figures 5.2 and 5.3), exhibiting maxima at 390 nm and minima at 420 nm. The amplitude measured between maximum and minimum absorbance was assumed to be proportional to the degree of substrate binding. The interaction of the extracts with P5 was also investigated – however neither the chloroform nor the methanol extract inhibited binding (results not shown).
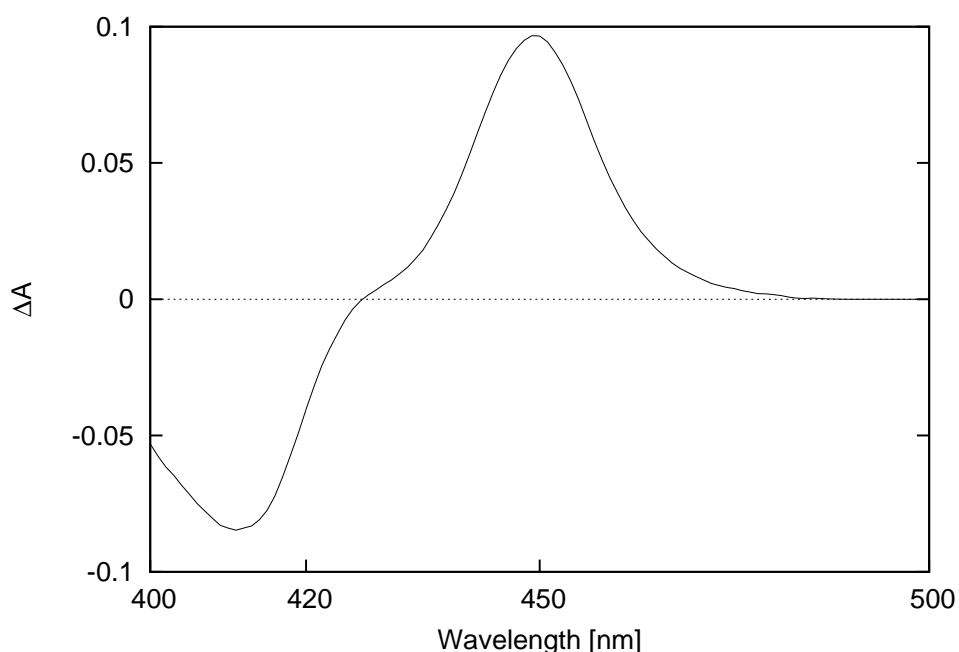
Figure 5.1: Sodium dithionite reduced carbon monoxide difference spectrum of the ovine adrenal microsomal preparation, [cytochrome P450] = 1.03 μM (0.155 nmol P450/mg of protein).

Due to plant pigments present in the extracts under investigation absorbing between 360 and 500 nm, as well as the turbidity of the microsomal suspensions, there was a certain amount of high-frequency noise in the recorded spectra. This was overcome by representing each spectrum as a seven-term Fourier series, using the FFTW algorithm in the Octave numeric analysis package. Typical smoothed curves are shown in figures 5.2 and 5.3.

P4 binding to microsomal P450 enzymes was inhibited by the chloroform extracts of both fermented and unfermented rooibos, with a greater degree of inhibition exhibited by the fermented rooibos (fig. 5.4). The same pattern was seen with the methanol extract, but because lower concentrations were used (1 mg/mL for the methanol extracts vs 2.4 and 2.5 mg/mL for the unfermented and fermented chloroform extracts, respectively), the degree of inhibition was not directly comparable. The difference between the fermented and unfermented extracts was not statistically significant at the 0.5% confidence level for either the methanol or chloroform extract.

17OH-P4 binding to microsomal P450 enzymes was also inhibited by both methanol and chloroform extracts of fermented and unfermented rooibos
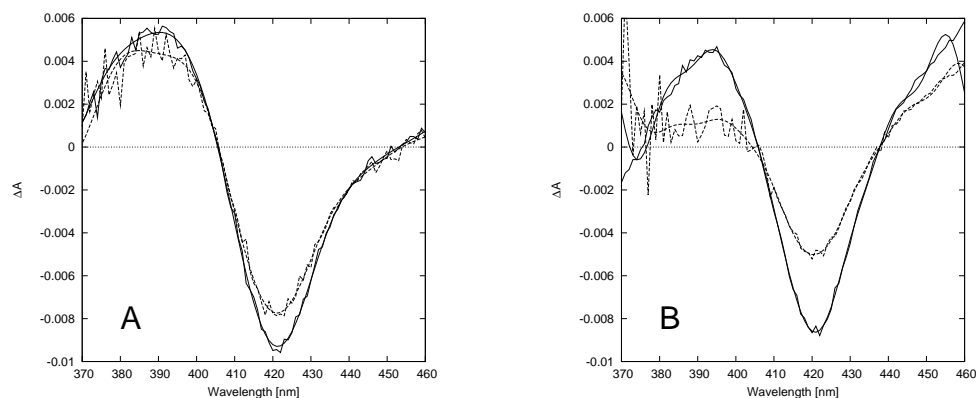
Figure 5.2: Inhibition of substrate-induced type I difference spectra in ovine adrenal microsomes by MeOH extract of unfermented rooibos, [cytochrome P450] = 0.3 μM. Substrate-induced difference spectra in the absence (—-) and in the presence (– –) of extract, A: P4 [3.2 μM], 2.4 mg extract/mL and B: 17OH-P4 [3.2 μM], 12.5 mg extract/mL; unprocessed curves shown together with Fourier smoothed data.
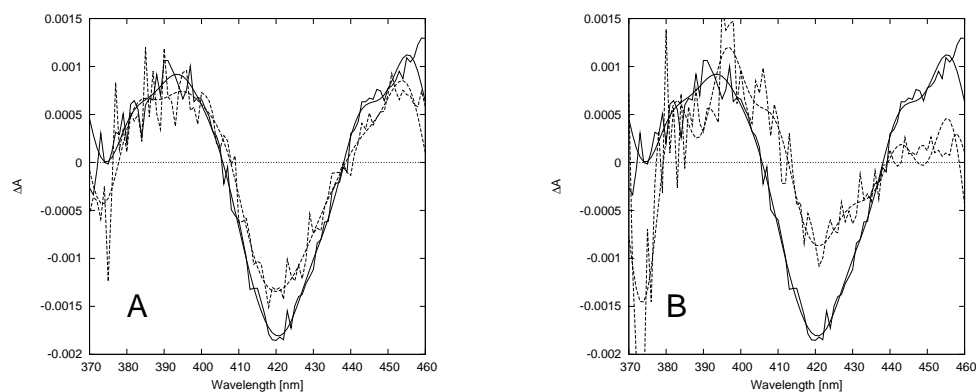
Figure 5.3: Inhibition of substrate-induced type I difference spectra in ovine adrenal microsomes by HIC column fractions, [cytochrome P450] = 0.3 μM; [P4] = 3.2 μM. Substrate-induced difference spectra in the absence (—-) and in the presence (– –) of extract, A: 0.29 mg hydrophilic fraction/mL and B: 0.145 mg hydrophobic fraction/mL; unprocessed curves shown together with Fourier smoothed data.
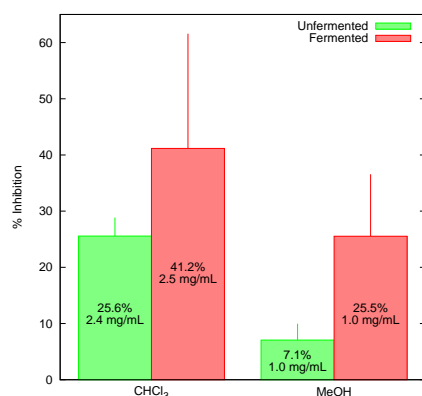
Figure 5.4: Percentage inhibition of P4 (3.2 μM) binding to adrenal microsomal P450 enzymes ([Cytochrome P450] = 0.3 μM) by unfermented and fermented rooibos chloroform extracts (2.4 and 2.5 mg/mL respectively) and by unfermented and fermented rooibos methanol extracts (final concentration 1.0 mg/mL; measurements in duplicate). Results are presented as the correlated value, error bars represent asymptotic standard error.

(fig. 5.5). As with the inhibition of P4, the differences between the fermented and unfermented extracts were not statistically significant, but in this case stronger inhibition was seen with the chloroform extract, and at slightly lower concentrations. To achieve comparable inhibition, a higher concentration of methanol extract was required – Methanol: 3.75 mg/mL vs the 1.2 mg/mL of the chloroform extract for the unfermented rooibos and 4 mg/mL vs the 1.25 mg/mL for the fermented rooibos.
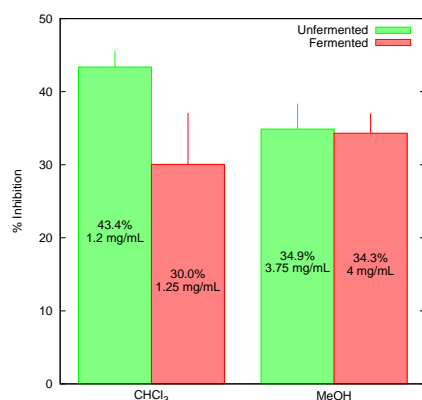
Figure 5.5: Percentage inhibition of 17OH-P4 (3.2 μM) binding to adrenal microsomal P450 enzymes ([Cytochrome P450] = 0.3 μM) by unfermented and fermented rooibos chloroform extracts (1.2 and 1.25 mg/mL respectively) and by unfermented and fermented rooibos methanol extracts (final concentration 3.75 and 4 mg/mL, respectively; measurements in duplicate). Results are presented as the correlated value, error bars represent asymptotic standard error.

Because of the different effects seen between the chloroform and methanol extracts, the HIC column fractions with the lowest and highest hydrophobicity (fraction 1 and fraction 5, respectively) were evaluated for their effect on P4 binding (fig. 5.6).

Both column fractions showed inhibition of P4 binding at a final concentration of 0.15 mg/mL. A stronger effect was seen with the more hydrophobic fraction, an observation which was consistent the effects of the chloroform
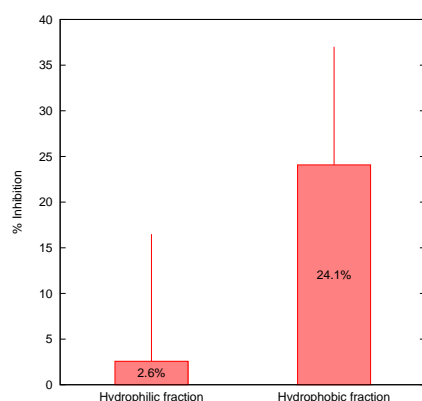
Figure 5.6: Percentage inhibition of P4 (3.2 μM) binding to adrenal microsomal P450 enzymes ([cytochrome P450] = 0.3 μM) by HIC hydrophilic and hydrophobic column fractions (final concentration 0.15 mg/mL; measurements in duplicate). Results are presented as the correlated value, error bars represent asymptotic standard error.

and methanol extracts. This difference was, however, not statistically significant (P > 0.2). The difficulty in achieving significant results can be largely ascribed to increased noise in the recorded spectra, due to the absorbance of the rooibos extracts. This had a negative impact on the repeatability of the experiments, despite data smoothing.

Determination of substrate binding inhibition parameters

The influence of methanol and chloroform extracts of fermented and unfermented rooibos on P4 and 17P4 binding to adrenal microsomal P450 was investigated. Two HIC column fractions, one more hydrophilic and one more hydrophobic, prepared from an aqueous extract of unfermented rooibos were also assayed with respect to their influence on the binding of P4 to microsomal P450.

Upon addition of increasing concentrations of substrate (P4 or 17OH-P4), type I substrate-induced difference spectra of increasing amplitude were observed. The relationship between the substrate concentrations and the measured amplitudes was well correlated by equation 5.2, confirming the assumption of first-order binding.

The graphs shown in figure 5.7–5.9 were not fitted individually to the data points through which they pass; a family of lines was calculated, with $[I]$ as the parameter distinguishing the individual lines. It can be seen from these plots that the mixed inhibition model used correlates well with the experimental data.

As previously mentioned, binding studies can only indicate the degree of inhibition of substrate binding, and cannot in general be used to distinguish between different types of mixed inhibition. While the competitive binding

inhibition constant, K_{si}, has direct relevance to competitive enzyme inhibition, the other parameter used in the mixed inhibition model, K_i, is simply an indication of a decrease in the total substrate binding capacity due to the presence of inhibitor. This can be due to reversible or irreversible modification of the enzyme such that the substrate cannot gain access to the active site, or due to an interaction involving the active site which causes the substrate not to interact normally with the heme group.

All the components of rooibos tea that had been used as standards during the HPLC investigation of the aqueous extracts of rooibos were also assayed to determine their effect on on P4 and 17OH-P4 binding to P450. Even at 100 μg/L, a much higher concentration than in rooibos tea, none of the pure compounds showed any effect on substrate binding (results not shown). The rooibos extracts and column fractions, however, showed marked inhibition of substrate binding, as can be seen from the summary of the competitive and non-competitive inhibition constants in table 5.1.

P4 and 17OH-P4 both bind to CYP17 and CYP21, so it is interesting that such a marked difference is seen between the form and degree of inhibition seen with P4 and that of 17OH-P4: with the exception of the chloroform extract of fermented rooibos, none of the extracts showed any competitive inhibition of 17OH-P4 binding, whereas in the case of P4, all the extracts tested showed mixed inhibition (a combination of competitive and non-competitive inhibitory effects).

The chloroform extracts of both unfermented and fermented rooibos showed stronger competitive inhibition than the corresponding methanol extracts, and the methanol extracts conversely showed stronger non-competitive effects than the corresponding chloroform extracts. This is not unexpected, in the light of the fact that the chloroform extracts would contain more hydrophobic substances than the methanol extracts, leading to better interaction with steroidogenic enzymes, which catalyse the metabolism of hydrophobic substrates.

Although neither of the column fractions showed any competitive inhibition of P4 binding, both showed strong non-competitive inhibition, with the more hydrophobic fraction (fraction 5) showing stronger inhibition.

Despite the general good correlation of the mixed inhibition model with the experimental data, anomalous results were seen in the competitive inhibition constants of the methanol extract of unfermented rooibos with respect to 17OH-P4 binding (fig. 5.7 B) and the hydrophobic column fraction with respect to P4 binding (fig. 5.9 B). Although a good correlation was seen with
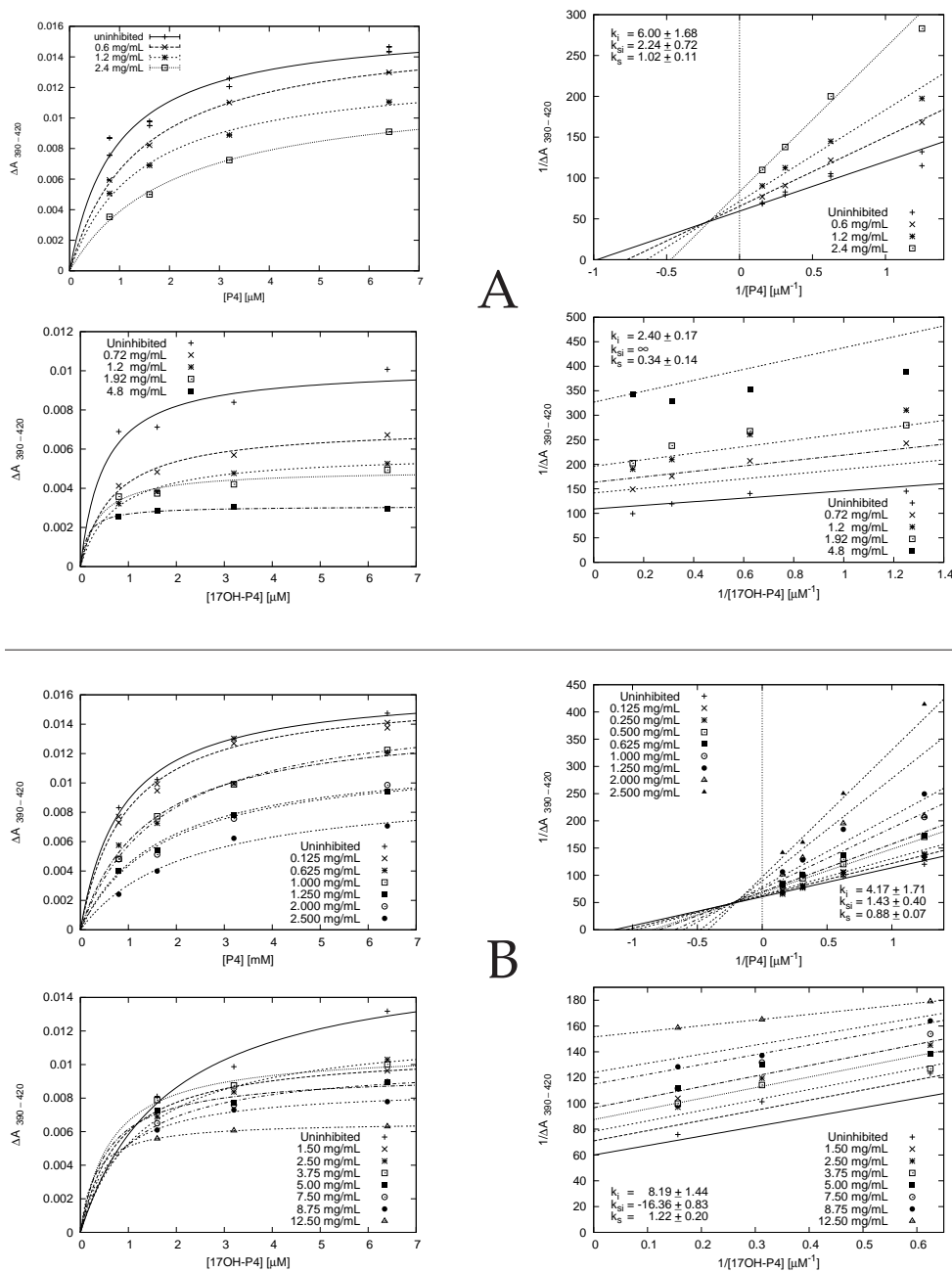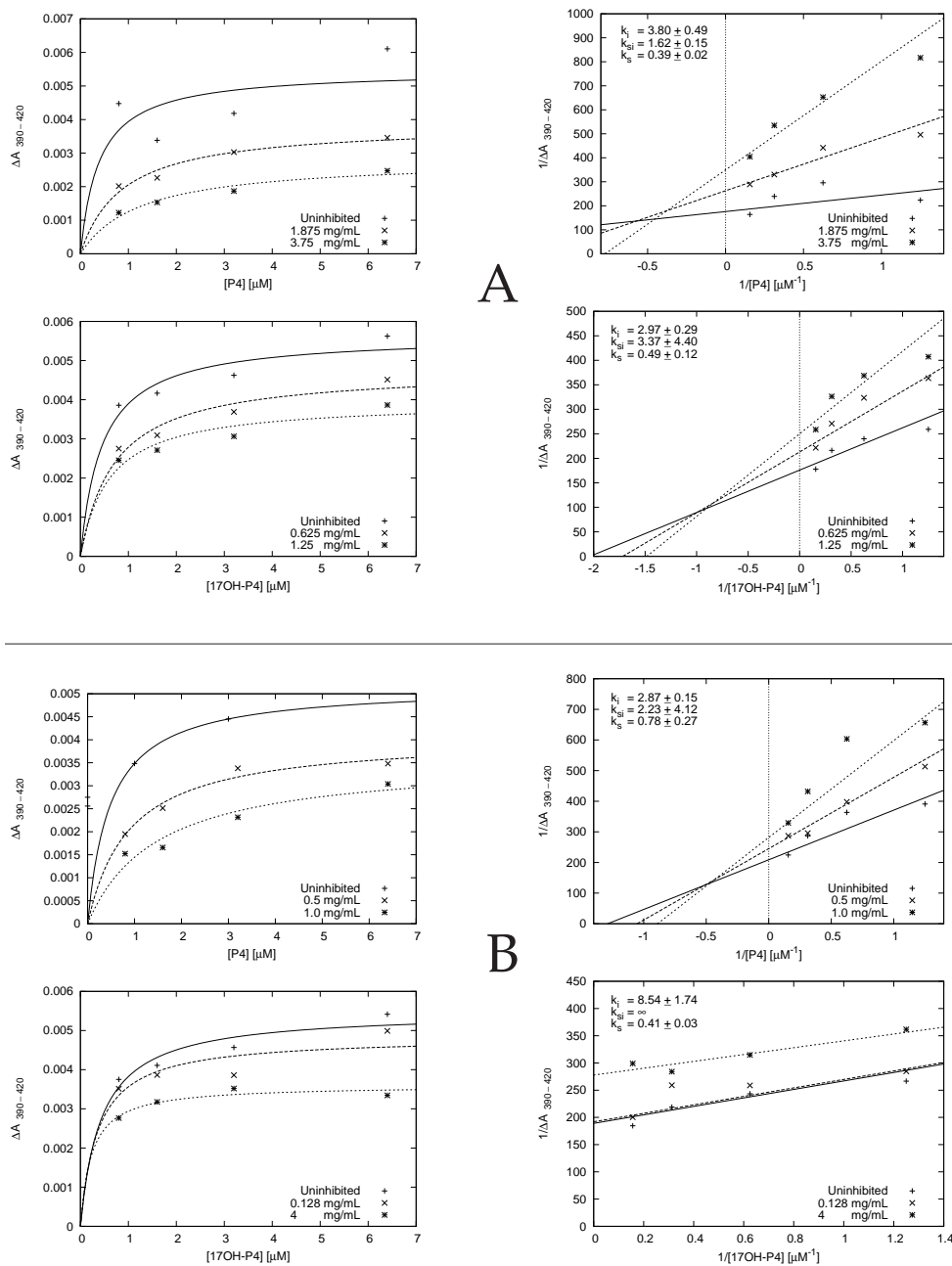
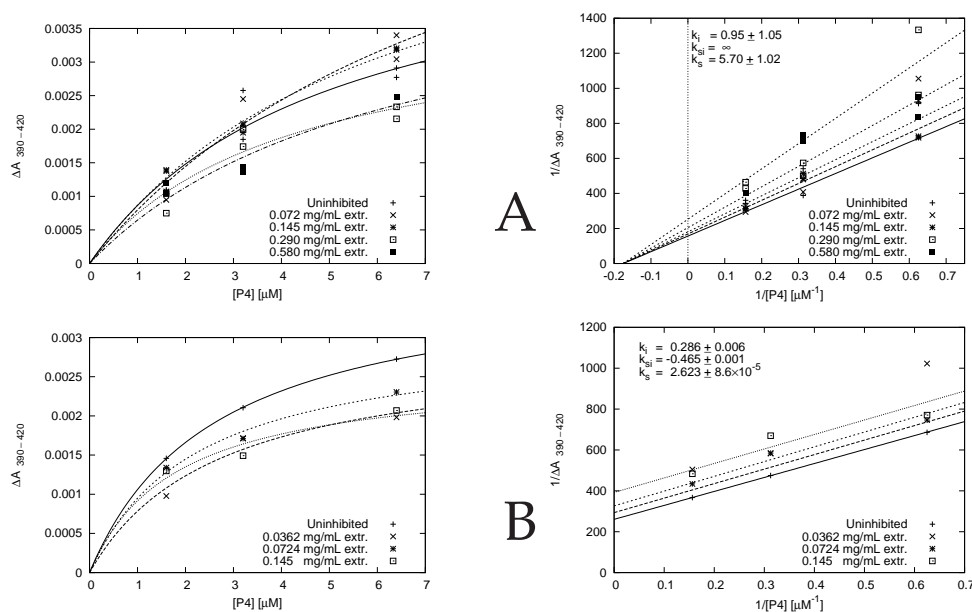**Figure 5.7:** Hyperbolic saturation binding curves and double-reciprocal plots of the inhibitory effect of unfermented rooibos extracts on P4 and 17OH-P4 binding to cytochrome P450 [0.3 μM]. **A**: CHCl$_3$ extracts (final concentration, 0.6 – 4.8 mg/mL), P4 and 17OH–P4 [0.8 – 6.4 μM]; **B**: MeOH extracts (final concentration, 0.125 – 12.50 mg/mL), P4 [0.8 – 6.4 μM], 17OH–P4 [1.6 – 6.4 μM]; measurements in duplicate.

Figure 5.8: Hyperbolic saturation binding curves and double-reciprocal plots of the inhibitory effect of fermented rooibos extracts on P4 and 17OH-P4 binding to cytochrome P450 [0.3 μM]. **A**: CHCl$_3$ extracts (final concentration, 0.62 – 3.75 mg/mL, P4 and 17OH-P4 [0.8 μM – 6.4 μM]); **B**: MeOH extracts (final concentration, 0.128 – 4.0 mg/mL, P4 and 17OH-P4 [0.8 μM – 6.4 μM]); measurements in duplicate.

Figure 5.9: Hyperbolic saturation binding curves and double-reciprocal plots of the inhibitory effect of unfermented rooibos HIC column fractions on P4 [1.6 μM – 6.4 μM] binding to cytochrome P450, [0.3 μM]. **A**: hydrophilic column fraction (final concentration 0.072 – 0.58 mg/mL); **B**: hydrophobic column fraction (final concentration, 0.036 – 0.145 mg/mL.; measurements in duplicate.

the experimental data, with the major effect in both cases being uncompetitive inhibition ($|K_i| < |K_{si}|$), the values of K_{si} were negative, theoretically indicating competitive activation. This is a rather improbable result, because activators are usually allosteric in nature, and usually either endogenous compounds (often metabolites relevant to the pathway in which the enzyme occurs) or compounds frequently encountered by the organism. It is quite probable, however, that the observed activation is an artifact of the data analysis method, because a numerically large value, negative or positive, indicates a weak effect. The fact that a better correlation was observed with a negative value should therefore not be taken as strong evidence of enzyme activation in this case.

5.4   Conclusions

In this chapter, an investigation of the effect of rooibos extracts and rooibos HIC column fractions on the binding of substrate to ovine adrenal microsomal P450 enzymes was described. Although, as discussed above, binding inhibition studies cannot measure inhibition effects which do not affect

Table 5.1: Inhibition parameters determined for various rooibos preparations with respect to the binding of P4 and 17OH-P4 to ovine adrenal P450. All parameters are reported as g/L plant material, except in the case of the column fractions, which are given as g/L dry powder.

		P4		17OH-P4	
		K_{si}	K_i	K_{si}	K_i
Unfermented	Chloroform	2.24 $\pm$ 0.72	6.00 $\pm$ 1.68	ND	2.40 $\pm$ 0.17
	Methanol	1.43 $\pm$ 0.40	4.17 $\pm$ 1.71	-16.36 $\pm$ 0.83	8.19 $\pm$ 1.44
Fermented	Chloroform	1.62 $\pm$ 0.15	3.80 $\pm$ 0.49	3.37 $\pm$ 4.40	2.97 $\pm$ 0.29
	Methanol	2.23 $\pm$ 4.12	2.87 $\pm$ 0.15	ND	8.54 $\pm$ 1.74
Column fraction	Hydrophilic [1]	ND	0.95 $\pm$ 1.05		
	Hydrophobic [2]	-0.465 $\pm$ 0.001	0.286 $\pm$ 0.006		

[1]fraction 1 in figure 3.5
[2]fraction 5 in figure 3.5

substrate binding, as in the case of pure non-competitive inhibition, such inhibitors are uncommon. Valuable information can, however, still be obtained from binding studies, both in terms of degree of inhibition, and also in terms of the type of inhibition.

As expected, the more hydrophobic extracts and the more hydrophobic column fractions had a stronger inhibitory effect on substrate binding. An unexpected result, however, was the difference between the type of inhibition observed in the binding of P4 and 17OH-P4. In ovine adrenal microsomes P4 is a substrate for both CYP17 and CYP21. Although 17OH-P4 is a substrate for CYP21, CYP17 is also able to bind 17OH-P4, as can be seen from the data represented here. It would be possible to determine the effect of extracts and column fractions on the individual enzymes using recombinant ovine CYP17 and CYP21 expressed in HEK 293 cells line, a non-steroidogenic mammalian kidney epithelial cell line where CYP17 exhibits lyase activity towards 17OH–P4. These investigations may contribute towards an understanding of the reasons for the differences observed in the inhibitory effects.

Because the extracts used in this study were only very broadly characterised mixtures of biological compounds, many of which still remain to be identified, further fractionation of rooibos extracts will be required to identify the

compounds or classes of compounds responsible for the effects seen. Considering the variety of types of inhibition seen, it is highly probable that multiple compounds are involved.

Because both CYP17 and CYP21 show a type I binding spectrum upon binding of P4 and 17OH-P4, *ex vivo* substrate binding experiments cannot identify which isoform is inhibited by rooibos extracts. However, if the metabolism of P4 to DOC and 17OH–P4 to S, which is catalysed by CYP21 were preferentially inhibited, this would decrease the flux towards the mineralocorticoids and glucocorticoids, and increase the flux towards the androgen precursors, which could conceivably mitigate the effects of an inappropriate stress response. Although this is a speculative conclusion, which needs to be confirmed by enzyme kinetic and *in vivo* studies, it is in line with the reputed health benefits of rooibos Tea.

As discussed above, although 17OH-P4 binds CYP17, it is not a substrate for CYP17 in *ex vivo* ovine adrenal preparations. Because 17OH-P4 is a natural substrate for CYP21, it is possible that the binding to CYP21 is less easily disrupted (see fig. 5.2). This would certainly influence the throughput through the steroidogenic pathways, but any such effects would have to be identified by substrate conversion studies. Conversion studies would also be needed to identify any effects which do not affect substrate binding, for example inhibition of binding of cofactors or purely noncompetitive effects.

Chapter 6

Conclusion

DOGBERRY: Marry, sir, they have committed false report; moreover, they have spoken untruths; secondarily, they are slanders; sixth and lastly, they have belied a lady; thirdly, they have verified unjust things; and, to conclude, they are lying knaves.
— *Much Ado About Nothing, Act V, scene I* (William Shakespeare)

The liberalization of international commodity markets and an international realization of the importance of ethical trade form a positive environment for the export of specialized local crops [75]. This is further bolstered by a receptive market for ethnic health products and natural products, especially when the health claims of such products can be scientifically supported.

Rooibos is ideally placed to take advantage of these factors, as a tenacious international marketing campaign over many years, supported by a growing body of research, has generated a positive reputation for the health benefits of rooibos tea. The scientific underpinnings of this marketing campaign have largely been based on the antioxidant properties of rooibos [1, 5, 29], with concomitant antimutagenic effects [3], which it has in common with a number of other natural products [31], including teas produced from *Camellia sinensis* [149]. Much is still unknown, however, about the minor constituents of rooibos and the subtle effects that these might have on the body, over and above their antioxidant effects. In the light of anecdotal reports of the utility of rooibos in stress-related conditions [34], its effects, if any, on the endocrine system merit investigation.

Polyphenols, especially those containing catechol moieties, have a combination of metal-chelating, antioxidant and radical-scavenging properties [150]. The phenylpropanoids, an important class of plant polyphenols, are synthesized in the shikimate pathway in plant metabolism [76]. Some of the major classes of phenylpropanoids include the tannins, lignins and flavonoids [76].

Tannins are well known as antinutritional factors, and claims that rooibos contains low levels of tannins [13] have therefore been favourable to the commercial development of the industry. However, more recent studies have shown rooibos to contain some tannins [88], with as much as 80% of the flavonoid fraction of rooibos tea consisting of tannins [87].

Plants use flavonoids for a variety of purposes, partly as substrates for synthesis of tannins, but also for their antioxidant and ultraviolet-absorbing properties. They are also particularly significant to human health [76]. Flavonoids, including flavonoids in rooibos tea, have been shown to have antimutagenic and anticarcinogenic effects, both *in vivo* and *in vitro*, with some of the published studies reporting results that are indicative of an effect involving the activity of liver P450 enzymes [3, 31]. Knowledge of the antioxidant effects of flavonoids is also becoming commercially important due to an increasing public awareness of the merit of antioxidants.

Since rooibos contains a complex mixture of flavonoids, fractionation of rooibos extracts is useful to their investigation. Rooibos extracts were therefore prepared using solvents of differing hydrophobicity, and aqueous rooibos extracts were subjected to column chromatography. The bioactivity of fractions having different characteristics and compositions was investigated.

HIC separation of aqueous fermented and unfermented rooibos into a more hydrophilic fraction and a more hydrophobic fraction showed a clear difference between the fractions produced in terms of polyphenol content as well as antioxidant capacity. A more comprehensive fractionation was therefore carried out, yielding a series of fractions of varying hydrophobicity, as was successfully visualized using RP-HPLC (Chapter 3).

The more hydrophobic fractions showed higher polyphenol concentrations and higher antioxidant activity than the hydrophilic fractions. These two parameters showed a linear correlation with each other. The fractions with the highest antioxidant capacity and polyphenol content were obtained from the unfermented rooibos, but while the antioxidant capacity of the more hydrophilic fractions of unfermented rooibos was negligible, the antioxidant capacity and polyphenol content of the more hydrophilic fractions of the fermented rooibos was markedly higher than the corresponding fractions of

unfermented rooibos. This could possibly be due to an increase in polarity during partial enzymatic oxidation of antioxidant molecules, but further analyses would be required to confirm this theory.

NP-HPLC analyses were also carried out in order to determine whether tannins could be identified in the extracts (Chapter 3). Although the regularly-spaced peaks characteristic of tannins were seen in this analysis, they were not as abundant as those observed in analyses of known tannin-rich materials previously published [108, 110]. Although the samples collected were insufficient for structural elucidation, molecular masses were determined using ESMS that placed the compounds well within the expected range for low-molecular-mass tannin species.

RP-HPLC was used to identify and quantify known rooibos polyphenols in both the aqueous extracts used in the HIC fractionation and a fraction of intermediate hydrophobicity, both of the fermented and the unfermented rooibos (chap. 3). The distribution of the compounds between the source extracts and the HIC fractions was well explained by their various hydrophobic and hydrophilic character.

Once the antioxidant capacity of the fermented and unfermented rooibos extracts had been established, together with its link to the polyphenol content, the influence of rooibos on steroidogenic P450 enzyme systems was investigated. The antioxidant compounds in rooibos have similar structures to the natural substrates of P450 enzymes, which would lead one to expect that these compounds might also interact with P450 enzymes.

P450 enzymes are a group of heme-containing enzymes that are located in the membranes of a variety of subcellular components [112]. Together with the electron-transport chains upon which they depend, they form so-called P450 enzyme systems which catalyze a variety of reactions, most notably mono-oxygenations of hydrophobic molecules [151]. An important family of P450 enzymes in the adrenal gland catalyzes reactions in the steroidogenic pathway. Unlike the P450 enzymes responsible for the metabolism of xenobiotic compounds in the liver, which catalyze the reaction of a wide variety of substrates and are inducible by increased substrate levels, the adrenal steroidogenic P450 enzymes have narrow substrate specificity, and their levels are under the control of hormones of the HPA axis [27].

The catalytic cycle of P450 enzymes is interrupted in the absence of reducing equivalents, as, for example, in isolated subcellular fractions. This fact, together with the spectral changes observed upon binding of substrates and inhibitors to P450 enzymes, allows for the convenient *in vitro* spectral assay of substrate binding to P450 enzymes [25].

Glucocorticoids participate in whole-body metabolism, and their levels are elevated during conditions of physical and psychological stress [27]. Inappropriate stress reactions can lead to chronic activation of the HPA axis and overproduction of cortisol, which in turn can precipitate a range of stress-related disorders [26, 124, 137].

Appropriate homeostatic control is dependent on the relative activities of CYP17 and CYP21, which direct the metabolism of steroidogenic intermediates towards either androgen precursors or glucocorticoids and mineralocorticoids [121–123]. While P4 is a natural substrate for both CYP21 and CYP17 in the ovine adrenal gland, 17OH-P4 is not a natural substrate for CYP17.

The catalytic activity of an enzyme is located in the active site [139], and enzymes are often dependent on prosthetic groups for their activity [139]. In the case of P450 enzymes, the catalytic site contains a heme prosthetic group and is located in a hydrophobic pocket within the enzyme structure [112]. There are specific structures involved in the entry of reactants and the exit of products to and from the catalytic site [112]. The activity of the enzyme is therefore vulnerable to disruption by molecules that either enter into the active site and render it unavailable to the natural substrate or by molecules that interfere with the entry of the substrate, the delivery of reducing equivalents to the active site or the release of products. These inhibitors can have reversible or irreversible effects, depending on their mode of action [139].

Although spectrophotometric methods for the analysis of P450 enzymes cannot distinguish inhibitory effects due to inhibitors that only exert their influence on the reaction step (i.e. after binding of substrate), they can still yield useful information on the degree and type of inhibition of substrate binding to the enzyme [25]. The two parameters that can be distinguished are (i) the influence on the half-maximal substrate binding concentration and (ii) the influence on the maximum degree of substrate binding. A mixed inhibition model was therefore applied.

Microsomal fractions containing a mixture of steroidogenic P450 enzymes were prepared from ovine adrenal glands, and P450 content was assayed to ensure consistent P450 concentration between experiments (Chapter 5). The effect of methanol and chloroform extracts of rooibos on P4 and 17OH-P4 binding to the microsomal P450 was determined, as was the effect of hydrophobic and hydrophilic HIC column fractions of unfermented rooibos on the binding of P4.

Pigments present in the extracts under investigation caused noise in the spectral data. Despite Fourier smoothing, this meant that no statistically significant differences were observed in the degree of inhibition seen between the

different extracts. However, the following trends were observed – the fermented rooibos extracts were more potent inhibitors of P4 binding than unfermented rooibos extracts, but a clear distinction could not be drawn between the effects of the methanol versus chloroform extracts. In the case of the inhibition of 17OH-P4 binding, however, the chloroform extracts had a stronger inhibitory effect, with the unfermented chloroform extract showing a stronger inhibitory effect than the fermented chloroform extract. In line with these results, the more hydrophobic column fraction had a stronger inhibitory effect on P4 binding.

By determining the inhibitory effect of different concentrations of extract on substrate binding, each over a range of substrate concentrations, the mixed inhibition model could be fitted to the results, yielding inhibition parameters. Although these inhibition parameters are based on the interaction of only partially characterised rooibos extracts on microsomal suspensions containing a mixture of P450 enzymes, trends were nonetheless observed: no competitive inhibition of 17OH-P4 was seen, with the sole exception of the chloroform extract of fermented rooibos. In contrast, both competitive and uncompetitive inhibition effects were seen with all the methanol and chloroform extracts of fermented and unfermented rooibos extracts in the case of P4 binding. Interestingly, the hydrophilic column fraction showed only non-competitive inhibition, while an anomalous result was seen with the hydrophobic fraction: the correlated competitive inhibition parameter was negative, indicating an activation, whereas the uncompetitive inhibition parameter was positive, indicating inhibition. This result is probably an artifact created by the algorithm used to fit the inhibition model being applied to data with an intrinsically low signal to noise ratio.

A number of major rooibos flavonoids were also subjected to substrate binding inhibition assays to determine whether they showed an inhibitory effect, but even far beyond physiologically relevant concentrations, no effect was seen. This indicates that other still to be identified components present in the rooibos extracts tested were responsible for the observed inhibitory effects.

Given the hydrophobic nature of the natural substrates of the P450 enzymes under investigation, it comes as no surprise that the more hydrophobic extracts and column fractions showed stronger inhibitory effects on substrate binding in this investigation. It is, however, interesting that such clear qualitative differences were seen between the type of inhibition demonstrated with P4 compared to that seen with 17OH-P4, given the fact that both CYP17 and CYP21 have the ability to bind either of these substrates. The significance of this finding deserves further study – investigations into the catalytic activity of these P450 enzymes in the microsomal environment as well as recombinant CYP17 and CYP21 in the presence of extracts or purified compounds would clarify the physiological relevance of these observed differences.

Although the work presented here does not quantify specific effects of rooibos constituents on the steroidogenic pathway, it does show that rooibos extracts have the capacity to affect the enzymes involved. This is consistent with the reported uses of rooibos tea in folk medicine, including hypertension, allergies, skin diseases, colic in infants, diabetes, liver diseases, insomnia and other sleep disorders, headaches, irritability, tension and mild depression [33, 34]. The degree and nature of inhibition of substrate binding to CYP17 and CYP21 by fermented and unfermented rooibos differs – indicating that the interaction with these enzymes cannot be attributed to a single compound. The research on rooibos to date has focussed on antioxidant and antimutagenic effects. These are certainly relevant to the health benefits of rooibos tea, but endocrine effects, possibly due to a different group of compounds, cannot be ruled out.

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Appendix A

HPLC chromatograms of analytical standards used in the analysis of Rooibos extracts.

Table A.1: Elution times and wavelengths of maximum absorbance of analytical standards used in the analysis of Rooibos extracts

compound name	Elution time [min]	Maximum absorbance wavelength [nm]
Aspalathin	22.03	288
Nothofagin	24.80	288
Isovitexin	23.40	338
Luteolin	29.78	372
Vitexin	23.07	338
Quercetin-3-β-D-glycoside	23.62	256
Quercetin dihydrate	29.77	256
Rutin Hydrate	22.95	256
3,4 DHBA	11.55	260
Caffeic acid	17.88	324
Ferulic acid	23.18	322
p-Coumaric acid	21.40	310
Vanillic acid	17.63	260
Syringic acid	18.83	274
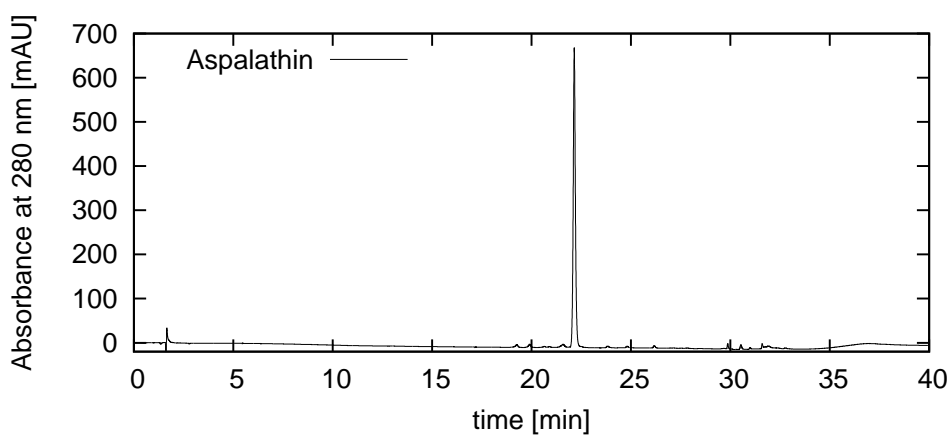
HPLC chromatogram of Aspalathin standard
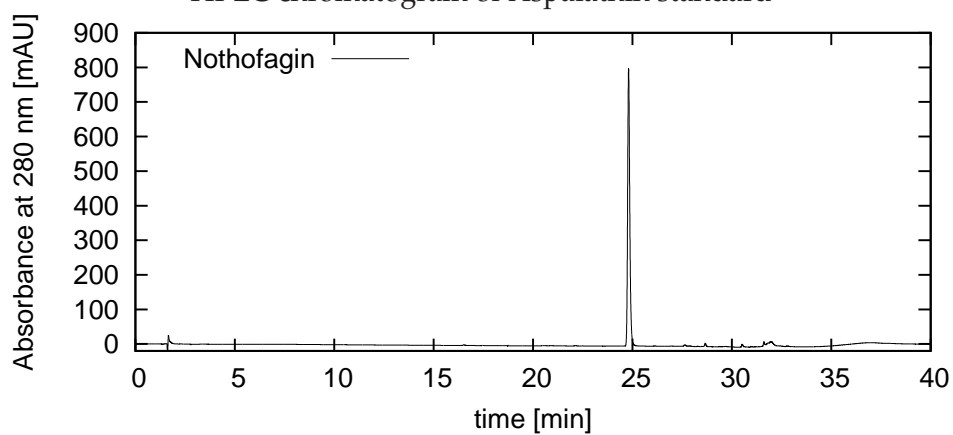
HPLC chromatogram of Nothofagin standard
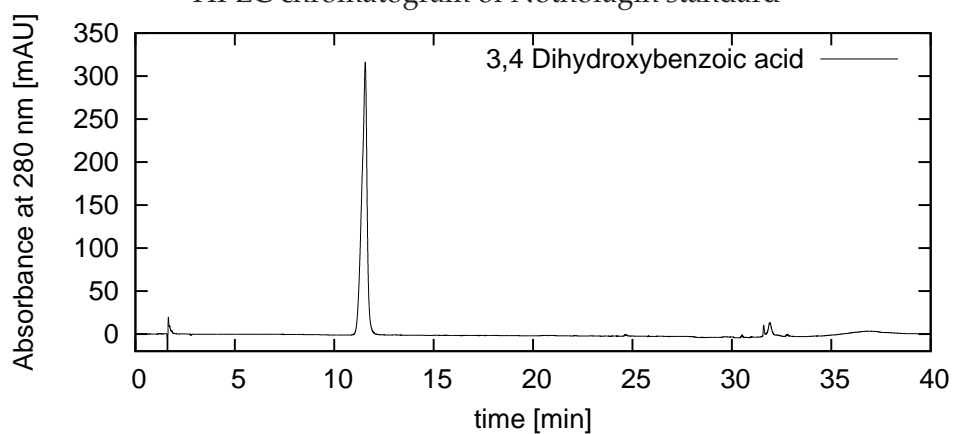
HPLC chromatogram of Dihydroxybenzoic acid standard
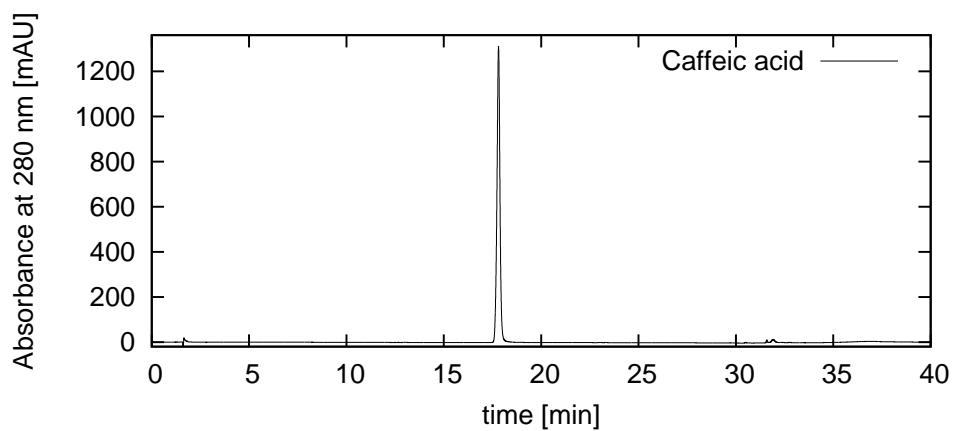
HPLC chromatogram of Caffeic acid standard
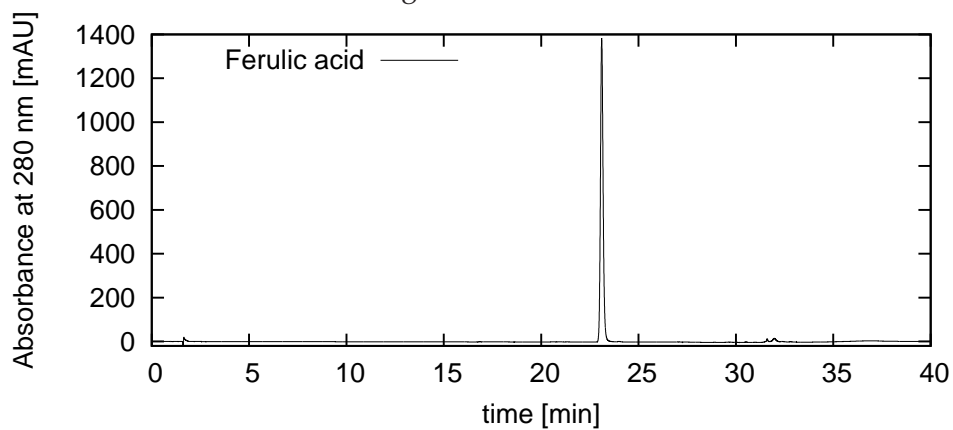
HPLC chromatogram of Ferulic acid standard
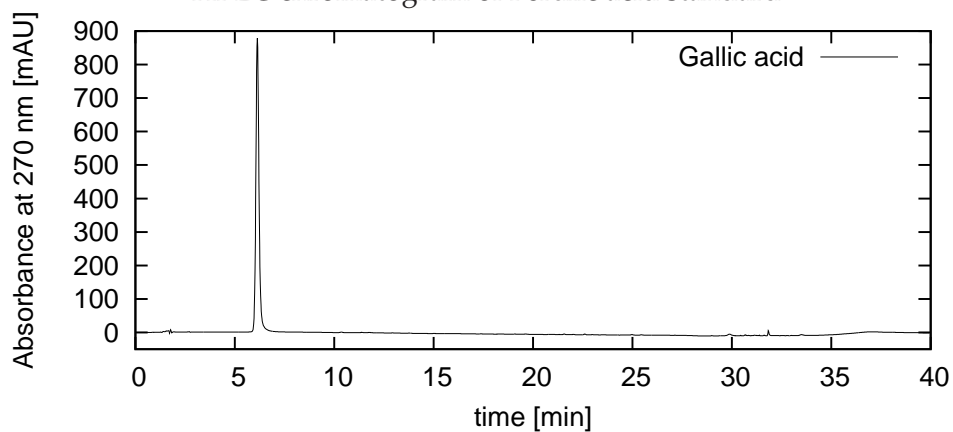
HPLC chromatogram of Gallic acid standard
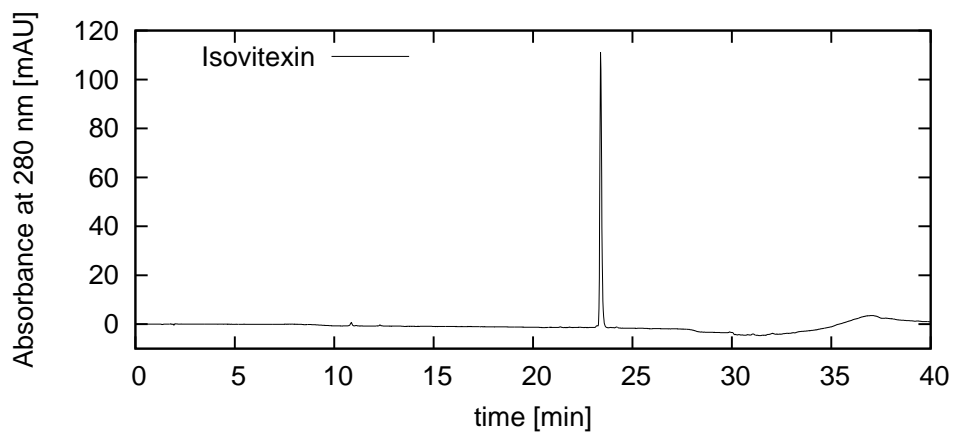
HPLC chromatogram of isovitexin standard
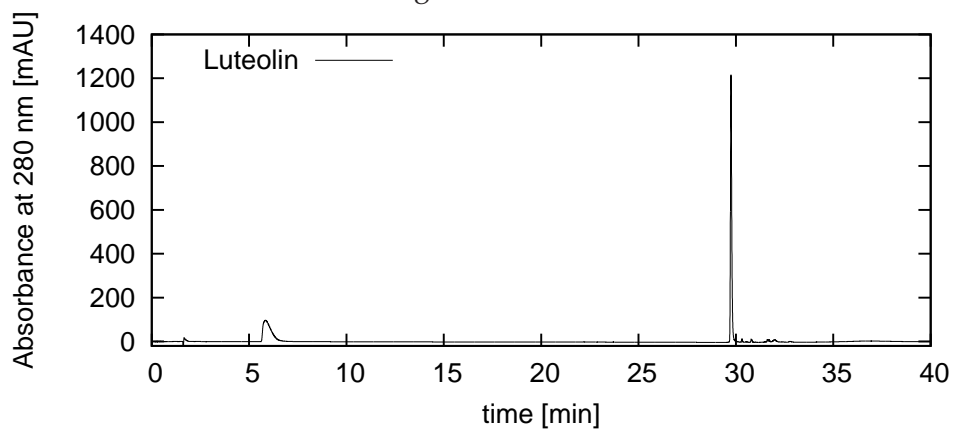
HPLC chromatogram of Luteolin standard
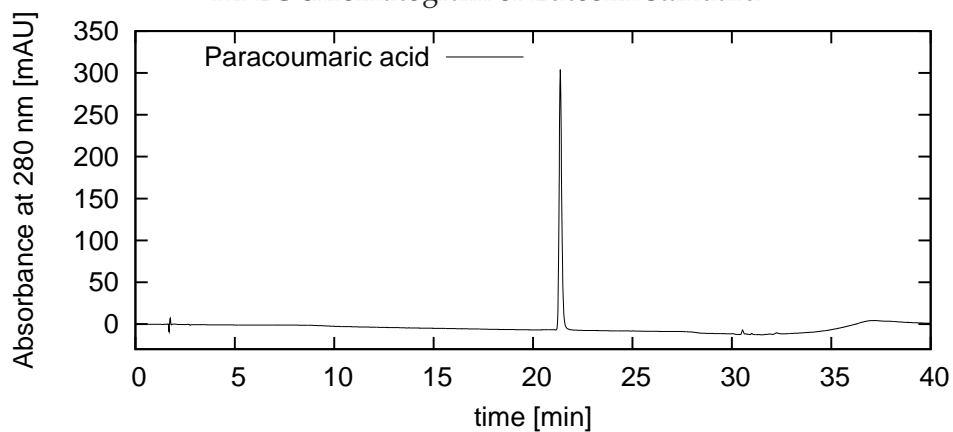
HPLC chromatogram of paracoumaric acid standard

HPLC chromatogram of Quercetin dihydrate standard

HPLC chromatogram of quercetin-3-β-D-glycoside standard
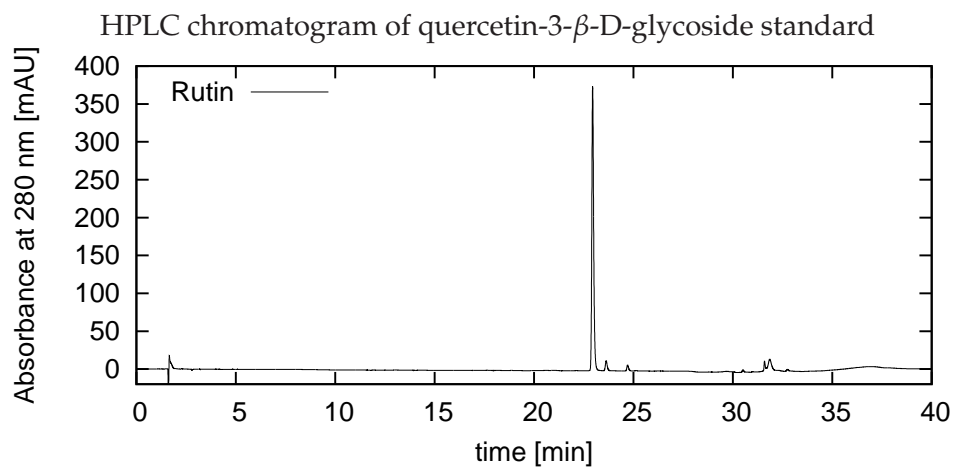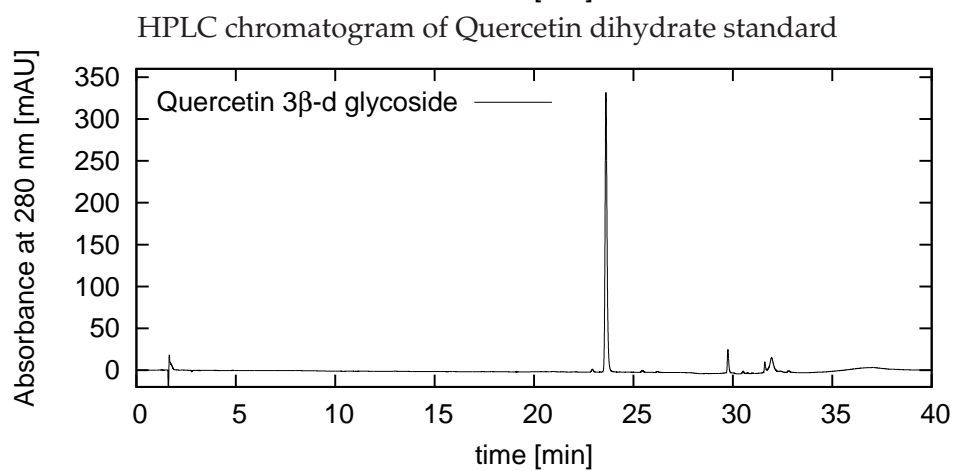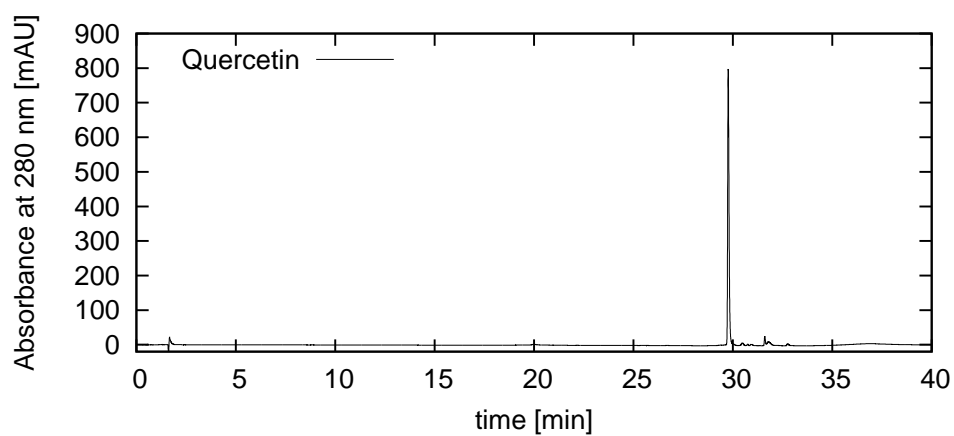
HPLC chromatogram of Rutin hydrate standard
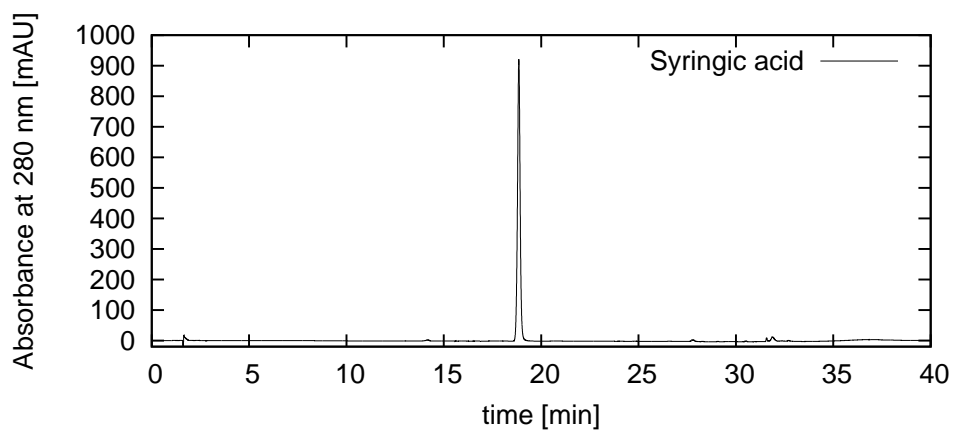
HPLC chromatogram of Syringic acid standard
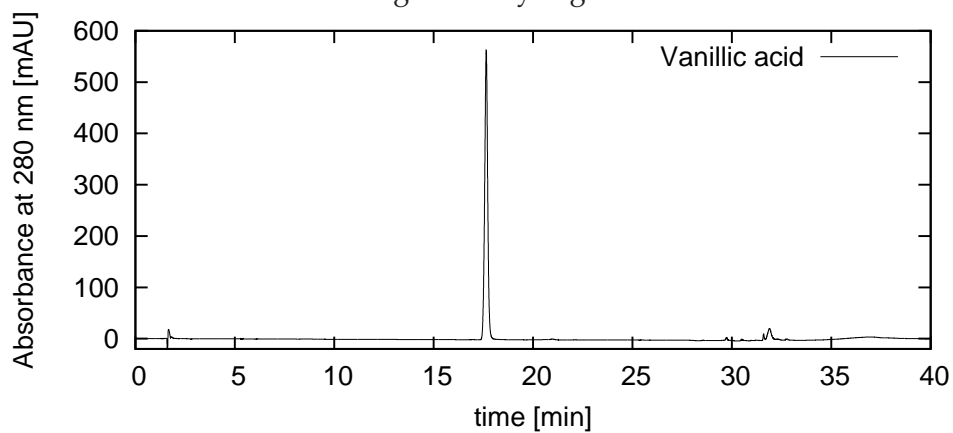
HPLC chromatogram of Vanillic acid standard
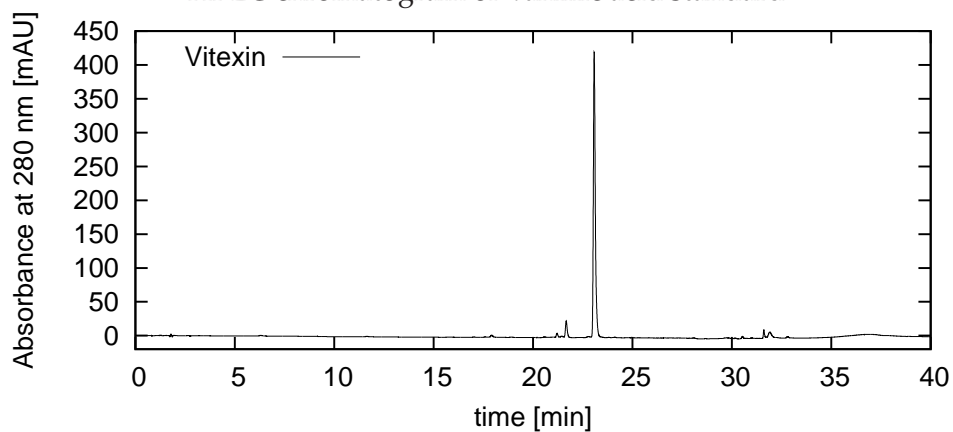
HPLC chromatogram of Vitexin standard

Appendix B

Ultraviolet spectra of standard compounds used in the analysis of rooibos extracts.
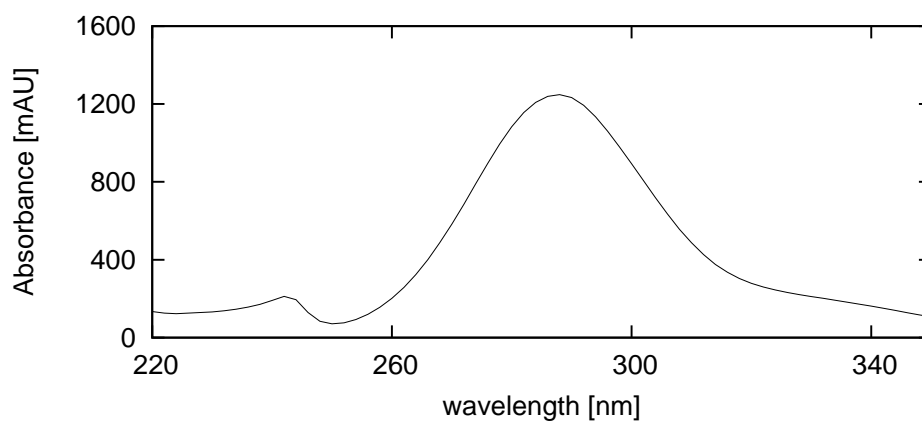
UV spectrum of Aspalathin standard
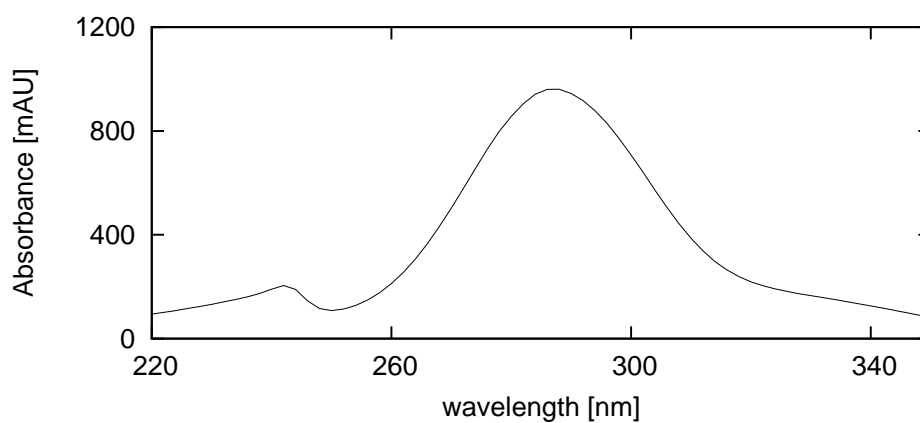
UV spectrum of nothofagin standard
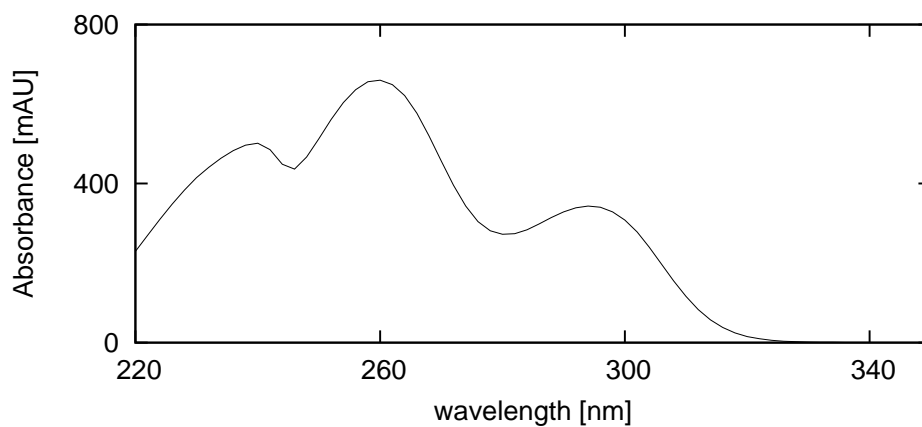
UV spectrum of 3,4 Dihydroxybenzoic acid standard
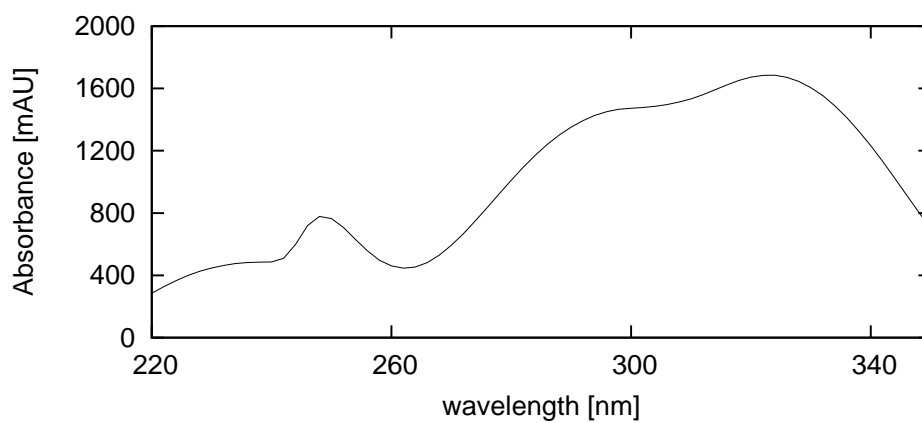
UV spectrum of caffeic acid standard
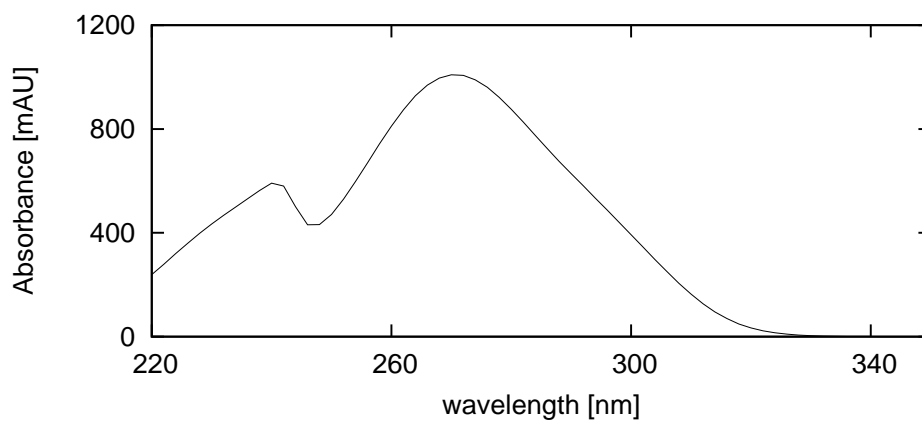
UV spectrum of gallic acid standard
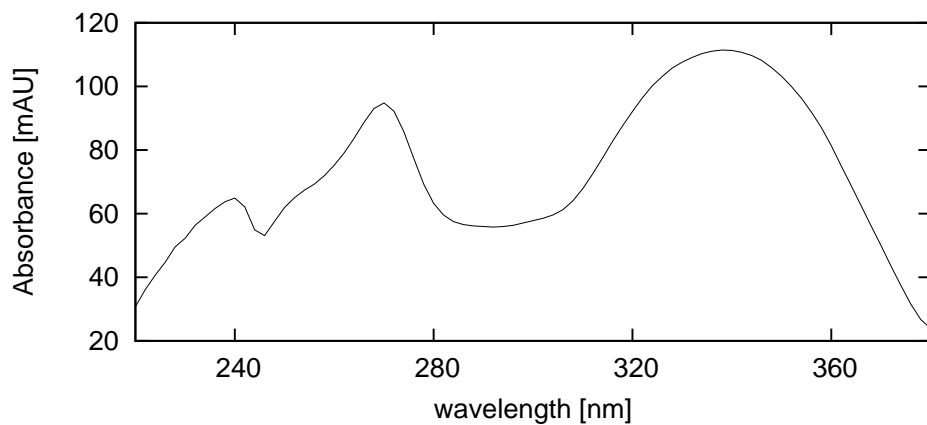
UV spectrum of isovitexin standard
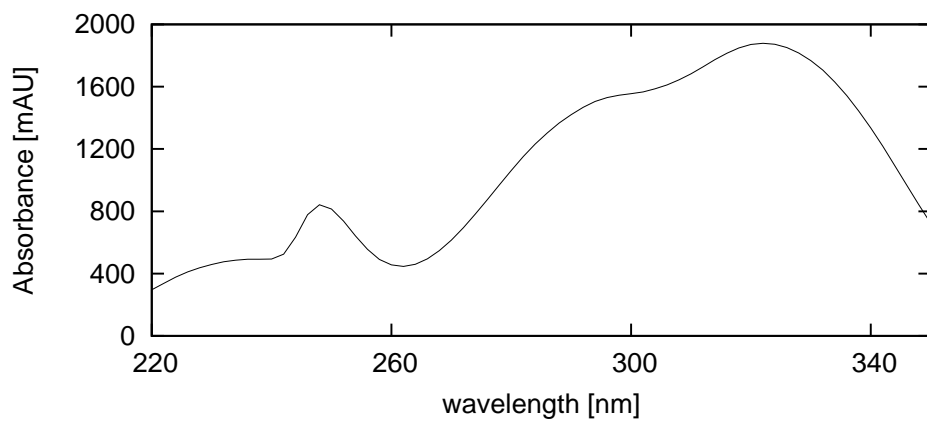
UV spectrum of ferulic acid standard
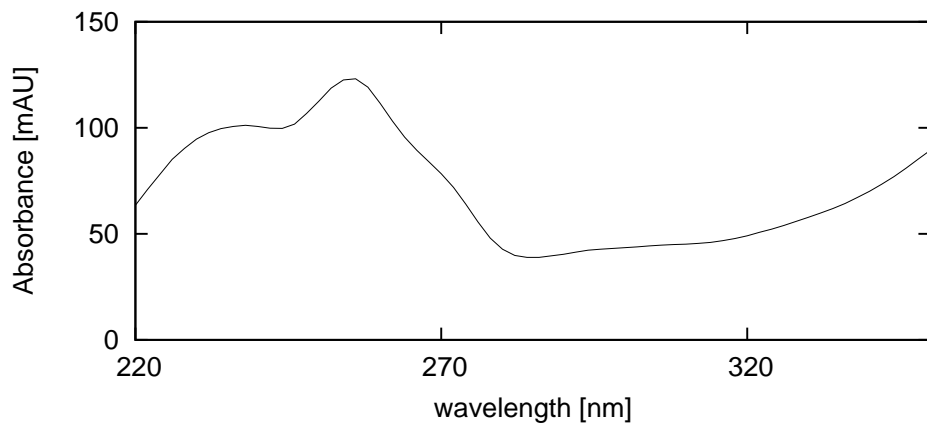
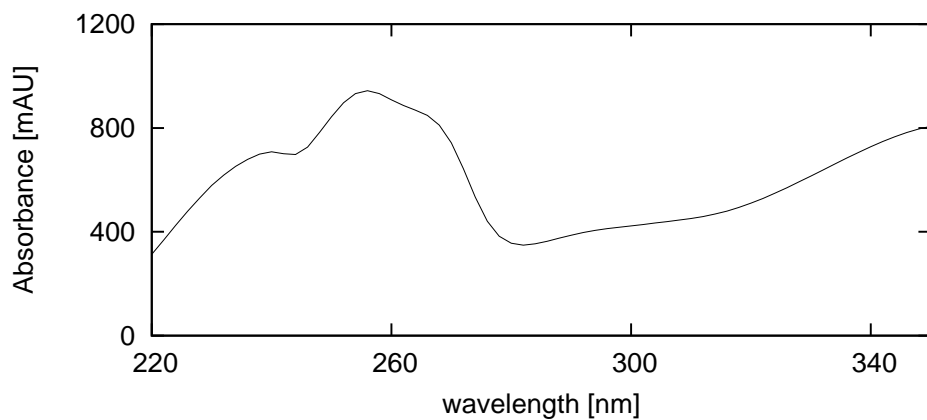

UV spectrum of luteolin standard
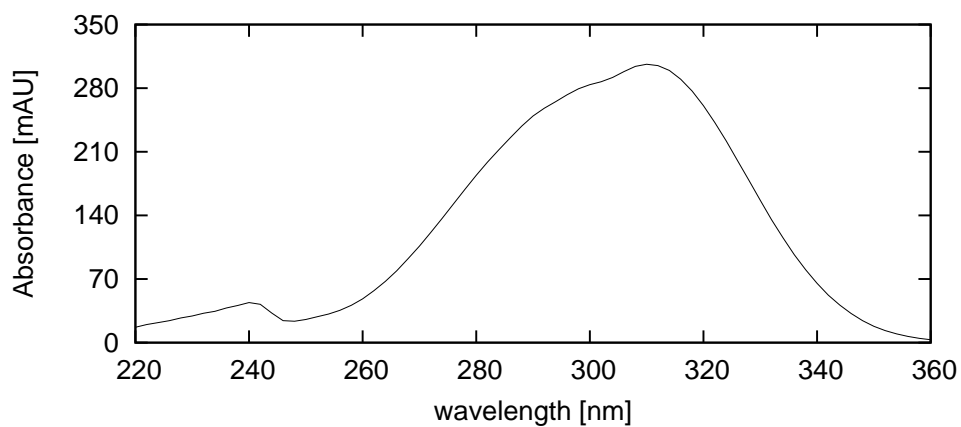
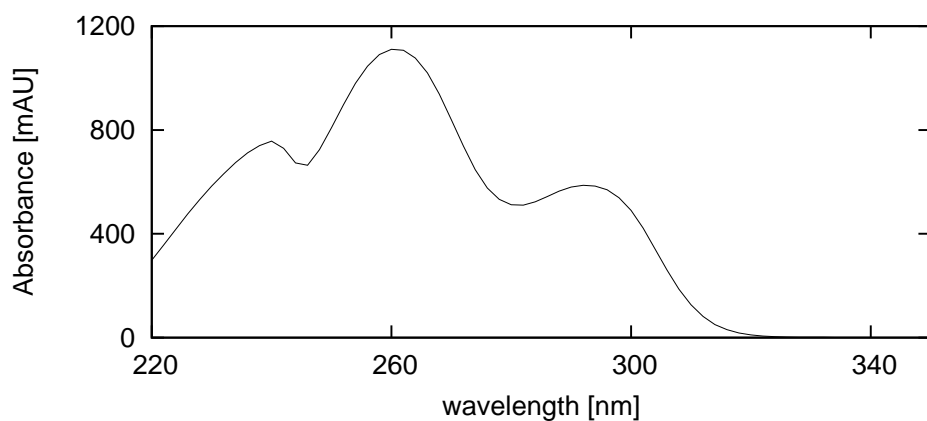UV spectrum of paracoumaric acid standard
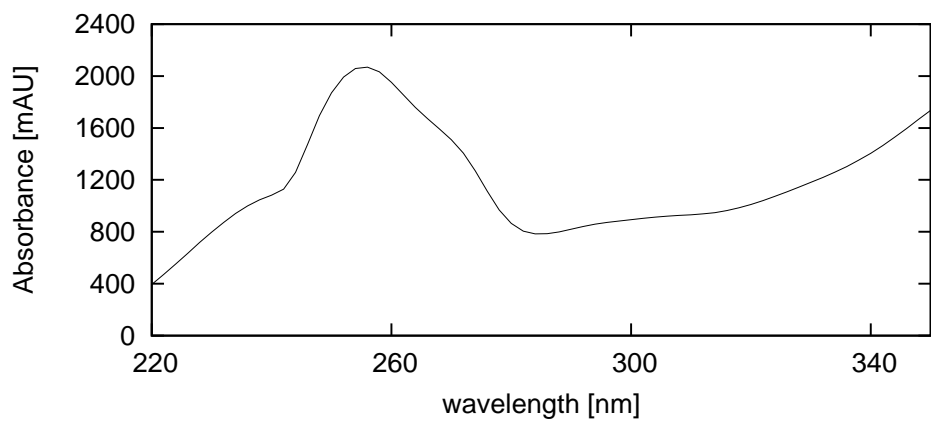
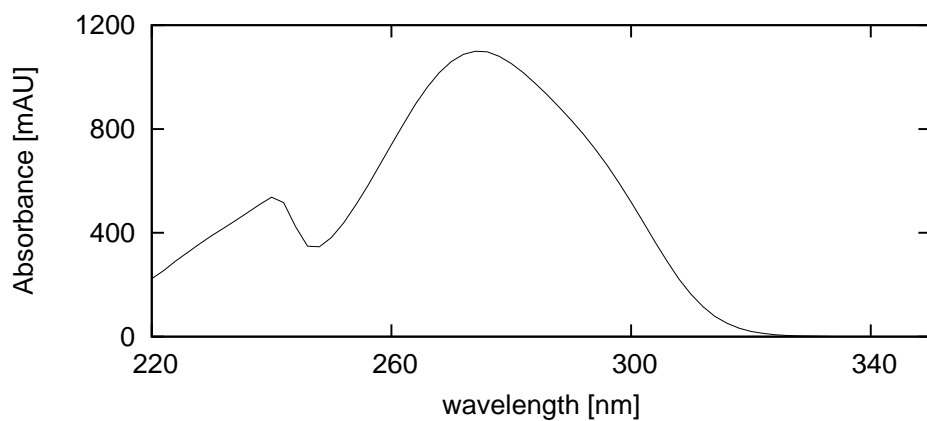UV spectrum of quercetin dihydrate standard
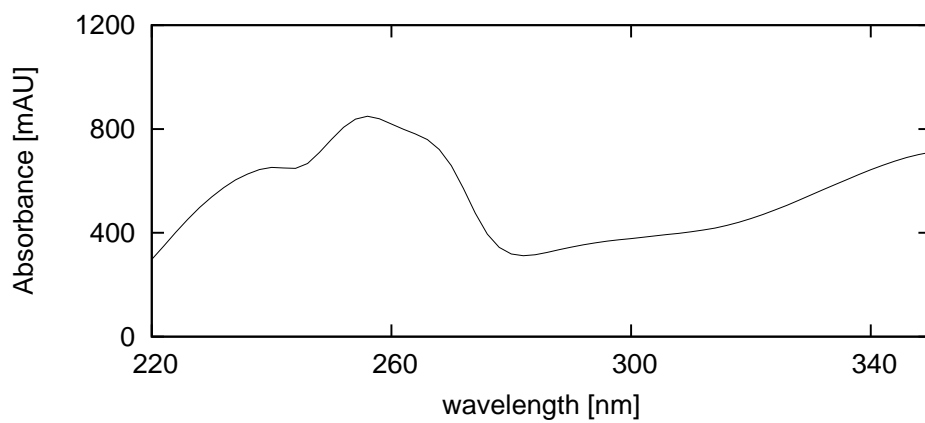
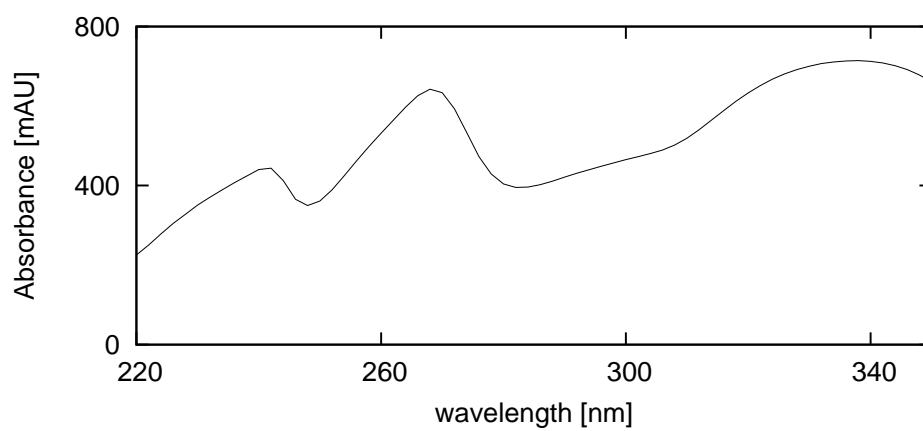UV spectrum of quercetin-3-β-D-glycoside standard

UV spectrum of rutin hydrate standard

UV spectrum of vanillic acid standard

UV spectrum of syringic acid standard

UV spectrum of vitexin standard

Appendix C

An investigation into the bioactivity of fermented and unfermented rooibos (*Aspalathus linearis*) extracts

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Stellenbosch 7602, South Africa
*Corresponding author

Abstract

Rooibos tea, a popular beverage made from *Aspalathus linearis*, known for its antioxidant properties, is also credited with stress-relieving effects, which may be due to effects on adrenal steroidogenic enzyme systems which catalyse glucocorticoid biosynthesis. In this study, the polyphenol content and antioxidant capacity of fermented and unfermented rooibos were enriched using hydrophobic interaction chromatography (HIC). Chloroform and methanol rooibos extracts, as well as HIC fractions of aqueous extracts, were subsequently investigated with respect to their effect on cytochrome P450-containing adrenal preparations. The influence of rooibos extracts, as well as aspalathin, nothofagin, isovitexin, luteolin, vitexin, quercetin-3-β-D-glycoside, quercetin dihydrate, rutin hydrate, 3,4 dihydroxybenzoic acid, caffeic acid, ferulic acid, p-coumaric acid, vanillic acid and syringic acid (polyphenols present in rooibos) on binding of progesterone (P4) to cytochrome P450 in ovine adrenal microsomal suspensions was assayed spectrophotometrically. Binding inhibition was observed for the extracts and column fractions, but not for the pure polyphenols. Mixed inhibition was seen with the methanol and chloroform extracts, with fermented rooibos exhibiting a stronger effect than unfermented rooibos. In contrast, the highest antioxidant capacity and polyphenol content were found in the hydrophobic column fraction of unfermented rooibos. Although the hydrophilic column fraction showed strong non-competitive inhibition, the hydrophobic fraction exhibited a stronger effect on P4 binding. These results show that, while unfermented rooibos has a greater antioxidant capacity, it is fermented rooibos that has a more potent inhibitory effect on adrenal cytochromes P450. Furthermore, the different types of inhibition manifested are evidence for the existence of more than one inhibitory compound.

1 Introduction

Rooibos tea, a herbal infusion made from *Aspalathus linearis* (N.L.Burm.) R.Dahlgr, has been enjoyed as a pleasant beverage since the beginning of the 20th century (*1*). Rooibos tea is native to South Africa, but it is exported worldwide (*2*) and is held in high regard for its perceived health-promoting properties. Besides not containing caffeine or high levels of tannin (*3*), it has been shown to act as an anti-oxidant (*4, 5*) and antimutagen (*6, 7*), and to stimulate the immune system (*8*).

The antioxidant and antimutagenic effects of rooibos are generally ascribed to its polyphenolic constituents, including i.a. aspalathin, nothofagin, rutin and quercetin (*9*). Polyphenols such as quercetin and rutin are known to have antioxidant and antimutagenic activity (*10–15*).

Rooibos tea is subjected to a so-called "fermentation" process after harvesting where the plant material is bruised and allowed to undergo oxidation in the open air to develop its characteristic colour, aroma and flavour. During this process, polyphenolic compounds, most notably aspalathin and nothofagin, are oxidised (*16*). Unfermented or green rooibos consequently has a higher antioxidative capacity than fermented rooibos (*17*).

Rooibos has also been claimed to be useful in the treatment of hypertension, allergies, skin diseases, colic in infants, diabetes, liver diseases, insomnia and other sleep disorders, headaches, irritability, tension and mild depression (*18, 19*). Although these claims have not been clinically investigated and are based on anecdotal evidence and folk wisdom, many are symptomatic of endocrine disorders that may possibly be precipitated by dysregulations of the stress response.

The stress response is associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing hormone (CRH), secreted by the hypothalamus, is carried by the hypothalamo-pituitary portal vessels to the anterior pituitary and stimulates adreno-corticotropic hormone (ACTH) release. ACTH stimulates the secretion of the glucocorticoid hormones, corticosterone and cortisol, by the adrenal gland (*20*). Adrenal glucocorticoids participate in the control of whole body homeostasis, regulating physiological processes. Prolonged activation of the HPA axis results in elevation of glucocorticoid levels, which in turn results in the inhibition of reproduction and growth, the inhibition of the inflammatory reaction and the inhibition of all the major components of the immune response, the promotion of visceral adiposity, insulin resistance, hypertension and "low turnover" osteoporosis. Elevated glucocorticoid levels are also associated

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with depression, chronic anxiety, fatigue and sleep disturbances (*21*).

In the adrenal cortex, cholesterol is converted to three classes of steroid hormones, viz. androgen precursors, mineralocorticoids and glucocorticoids. Most of the enzymes responsible for catalysing these reactions are members of the cytochrome P450 family, including Cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17) and cytochrome P450 21-hydroxylase (CYP21). These two enzymes stand at the branch point of glucocorticoid and androgen biosynthesis, and their relative activities therefore determine the outcome of steroid hormone biosynthesis. CYP17 catalyses the conversion of pregnenolone (P5) and P4 to 17-hydroxypregnenolone (17OH-P5) and 17-hydroxyprogesterone (17OH-P4), respectively. CYP17 subsequently metabolises these intermediates to androgen precursors. CYP21 however, channels P4 into the pathway which leads to the mineralocorticoid, aldosterone, and 17OH-P4 into the pathway which leads to the glucocorticoids, cortisol and corticosterone.

Steroidogenic P450 enzymes are highly specific for their natural substrates and consequently compounds which inhibit the binding of substrates could result in a change in the outcome of steroid hormone biosynthesis, which can significantly affect physiological processes. The activity of CYP17 in particular is crucial to the balance of androgen precursors, mineralocorticoids and glucocorticoids produced by the adrenal. We have previously shown that compounds isolated from the shrub *Salsola tuberculatiformis botch*, which causes prolonged gestation in karakul sheep, inhibit cytochrome P450 enzymes which catalyse the biosynthesis of cortisol (*22*).

The aims of this study were, firstly, to investigate the effect of HIC inrichment on the polyphenol content and antioxidant capacity of fermented and unfermented aqueous rooibos extracts, and then to investigate the effect of fermented and unfermented rooibos extracts and HIC fractions on the binding of CYP17 to its substrate P4. The influence of isolated phenolic compounds, known to occur in rooibos, on CYP17 substrate binding was investigated to ascertain whether the influence of rooibos on adrenal steroidogenesis could be attributed to the major polyphenols present in rooibos.

2 Materials and methods

2.1 Materials and equipment

Aqueous extracts of fermented and unfermented rooibos (technical grade), finely ground unfermented rooibos and tea bag cut fermented rooibos were provided by Benedict Technology Holdings (Pty) Ltd., Stellenbosch, South Africa. Rutin was purchased from Merck (Darmstadt, Germany). Aspalathin and Nothofagin were purchased from the Medical Research Commission of South Africa, and 3,4 Dihydroxybenzoic acid (DHBA, protocatechuic acid), caffeic acid, luteolin, vitexin, quercetin 3-β-D-glucoside, quer-

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cetin dihydrate, ferulic acid, rutin hydrate, syringic acid, vanillic acid and p-coumaric acid were purchased from Sigma-Aldrich (St Louis MO USA). HPLC spectra were recorded using a ThermoSep SpectraSystem consisting of a P4000 solvent delivery system, an AS3000 autosampler and column oven and a UV6000LP diode-array detector. The system was controlled by a Windows[TM] 2000 workstation running ThermoQuest[TM] software. HIC columns were supplied by Benedict Technology Holdings (Pty) Ltd., South Africa. Millipore syringe filters were purchased from Microsep (Pty) Ltd., South Africa. Difference spectra were recorded using a Cary 100 double-beam spectrophotometer.

2.2 Liquid chromatography of aqueous rooibos extracts

Immediately prior to use, the aqueous rooibos extracts were centrifuged at $7\,500 \times g$ for 30 minutes. The supernatants were subsequently fractionated using hydrophobic interaction chromatography (HIC) – five fractions of the unfermented rooibos and five fractions of the fermented rooibos extract were collected. The fractions were dried under reduced pressure with a rotating evaporator (Büchi, Switzerland) and lyophilized prior to further analysis.

The lyophilized samples were redissolved in deionized water to a concentration of 1 mg/mL and centrifuged on a low-speed benchtop centrifuge. The insoluble pellet was discarded, and the supernatant was filtered using a 20 μm filter.

2.3 Determination of polyphenol content and antioxidant capacity of HIC fractions

Polyphenols in fermented and unfermented rooibos HIC fractions were determined as described by Singleton and Rossi (23). Assays were carried out according to the Folin-Ciocalteu method using gallic acid as a standard. Briefly, the sample (200 μL) was mixed with 7.5% Na_2CO_3 (800 μL) and Folin-Ciocalteu's reagent (1000 μL) and incubated at 30 °C for 90 minutes. The absorbance was subsequently measured at 765 nm. Results are expressed as % gallic acid equivalents (%GAE).

Antioxidant capacities of fermented and unfermented rooibos HIC fractions were determined according to the α, α-diphenyl-β-picrylhydrazyl (DPPH) scavenging method, by monitoring the decrease in the DPPH concentration at 515 nm, as described by Brand-Williams et al. (24).

2.4 Preparation of chloroform and methanol extracts of rooibos

Unfermented rooibos plant material, 25 g, was subjected to continuous Soxhlet extraction with chloroform. Once the effluent from the extraction vessel was clear, the remaining plant material was extracted with methanol.

The extracts were dried at reduced pressure in a rotating evaporator. The dried chloroform extract was redissolved in 30 mL of propylene glycol, and the dried methanol extract was redissolved in 50 mL of deionized water. Fermented rooibos tea, 25 g, was subjected to chloroform and methanol extraction in the same manner, with the dried chloroform extract redissolved in 25 mL of propylene glycol and the dried methanol extract redissolved in 25 mL of deionized water. Extracts were centrifuged prior to being assayed for bioactivity in adrenal microsomal preparations containing CYP17 and CYP21.

2.5 Identification and quantification of rooibos polyphenols

Fermented and unfermented rooibos extracts and reconstituted HIC column fractions were subjected to high performance liquid chromatography with diode-array-detection (HPLC-DAD) to identify and quantify polyphenolic compounds. HPLC-DAD allowed for detection of substances with different UV absorbance maxima as well as peak identification by comparison of elution times and UV absorbance spectra with those of known standards.

Fresh rooibos tea extracts were filtered using 20 μm syringe filters immediately prior to use. Analytical standards were made up to a concentration of 10 μg/mL in ethanol or purified water.

Reverse phase HPLC (RP-HPLC) was carried out using a Zorbax SB-C18 column, particle size 3.5 μm, under the following conditions, using a linear gradient:

Solvent A: 1% formic acid in water
Solvent B: 100% acetonitrile
Column temperature 35 °C

Time [min]	% A	Flow rate [mL/min]
0	100	0.5
4	100	0.5
25	75	0.5
30	40	0.5
30.1	40	0.7
35	100	0.7
40	100	0.7

Chromatograms were recorded using a diode array detector, set to monitor the eluent over the wavelength range 200-400 nm.

2.6 Preparation of cytochrome P450 containing microsomal preparations

Microsomes were prepared as described by Yang and Cederbaum (*25*), using fresh ovine adrenal glands. The adrenals were decapsulated and homogenised in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.25 M sucrose and centrifuged for 20 min at $1\,000 \times g$. The supernatant fraction was centrifuged for 15 min at $12\,000 \times g$. Polyethylene glycol 800 (PEG), 50% (w/v) was added to the supernatant (final concentration, 8.5%), stirred for 10 min at $4\,°C$ and centrifuged for 20 min at $13\,000 \times g$.

The pellet was resuspended and homogenised in 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl and 1 mM EDTA. PEG was added to the suspension to a final concentration of 8.5%, stirred for 10 min at $4\,°C$, and subsequently centrifuged for 20 min at $13\,000 \times g$. This procedure was repeated twice, and the final microsomal pellet was re-suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.25 M sucrose. The P450 concentration was determined as described by Omura and Sato (*26*). The enzyme preparation was saturated with CO and reduced with sodium dithionite. A millimolar extinction coefficient of $91\ \frac{\text{AU}}{\text{cm·mM}}$ was used to calculate the P450 concentration (*26*).

2.7 Binding and inhibition assays

Microsomal preparations were diluted with 0.1 M phosphate buffer, pH 7.4, containing 10% ethylene glycol, to a final P450 concentration of 300 nM. Substrate induced difference spectra were recorded between 360 and 500 nm in the presence of the steroid substrate P4, using concentrations ranging from 0.8 to 6.4 μM P4, to generate saturation binding curves.

Inhibition of substrate binding was determined in the presence of rooibos extracts and pure phenolic compounds.

The reconstituted rooibos extracts, column fractions and phenolic compounds were added to microsomal preparations and incubated at room temperature for 5 minutes, after which saturation substrate binding curves were determined as described above.

Curve fitting was done using an implementation of the Marquardt-Levenberg nonlinear least-squares algorithm in the Gnuplot analysis package (*27*). The asymptotic standard errors of the calculated parameters were used as a measure of goodness of fit.

3 Results and discussion

3.1 Analysis of rooibos polyphenols

The HIC fractions of the fermented and unfermented rooibos were assayed for polyphenol content and antioxidant capacity. The later, more hydrophobic fractions were richer in polyphenols than the earlier, more hydrophilic fractions, and also had higher radical-scavenging activity (fig. 1 and fig. 2). The correlation between the radical-scavenging capacity and polyphenol content was consistent between the fermented and unfermented rooibos fractions, and was well described by a linear equation passing near the origin of the graph (fig. 3). This is consistent with current research, as the antioxidant capacity of rooibos is believed to be primarily due to its polyphenol content. The fact that the fractions with the highest total antioxidant activity and polyphenol content were prepared from the unfermented rooibos is consistent with the results reported for aqueous extracts by Standley *et al.* (*17*).

Since the difference between the polyphenol content of the hydrophilic and hydrophobic fractions of the unfermented rooibos was greater than that of the fermented rooibos, the most hydrophobic fraction and most hydrophilic fraction of the unfermented rooibos were selected for further investigation. A representative chromatogram of each of these fractions is shown in figure 4, along with a chromatogram of the unfermented rooibos extract from which they were produced. Aspalathin, nothofagin, 3,4 dihydroxybenzoic acid, caffeic acid, luteolin, vitexin, quercetin 3-β-D-glucoside, quercetin dihydrate, ferulic acid, rutin hydrate, syringic acid, vanillic acid and *p*-coumaric acid standards were prepared and subjected to HPLC analysis in order to generate calibration curves. Once the calibration curves had been generated, unfermented and fermented rooibos extracts and HIC column fractions of intermediate hydrophobicity derived from each of the rooibos extracts were analysed (table 1). As reported by Joubert (1995), both aspalathin and nothofagin were substantially reduced in the fermented rooibos relative to the unfermented rooibos. This difference was reflected in the corresponding HIC fractions. Minor rooibos components chrysoeriol, isoquercitrin and paracoumaric acid were not detected.

3.2 Binding and inhibition assay – difference spectra

All P450 enzymes have a protoporphyrin IX nucleus in their active sites. The heme iron is tetracoordinated between four pyrole rings leaving the 5th and 6th (axial) ligands open for interaction. The interaction of the iron atom with the fifth axial ligand, a cysteine derived S$^-$, results in the unique spectral properties of these enzymes observed in the Sorret region (*28*). Upon binding to substrates or inhibitors, the P450 enzymes exhibit type I, type II or modified type I induced difference spectra. These spectra have charac-

teristic, specific absorbance maxima and minima. Binding of substrates or substrate analogues induces either the type I difference spectrum (with an absorbance maximum at 385 - 395 nm and an absorbance minimum at 420 nm) or the modified type I difference spectrum (with spectral changes that are opposite to the type I spectrum), while inhibitors that bind directly to the iron atom give rise to the type II difference spectrum (*29*). In the absence of reducing equivalents from the electron transport chain, the substrate remains bound to the active site, and does not undergo conversion. The amplitude of the resulting binding spectrum can be used as a measure of substrate binding (*26*).

On addition of P4 to the microsomal preparations, type I substrate-induced difference spectra were observed (figures 5 and 6), exhibiting maxima at 390 nm and minima at 420 nm. The amplitude measured between the maximum and minimum absorbance is proportional to the degree of substrate binding.

Due to plant pigments in the extracts also absorbing between 360 and 500 nm and the turbidity of the microsomal suspensions, there was a certain amount of high-frequency noise in the recorded spectra. This was overcome by representing each spectrum as a seven-term Fourier series, using the FFTW algorithm in the Octave numeric analysis package (*30*). Typical smoothed curves, in the presence and absence of rooibos extracts, are shown in figures 5 and 6.

In the presence of the rooibos extracts and HIC column fractions, the type I binding curves were observed to be attenuated (fig. 7 and 8). Equilibrium binding studies were subsequently undertaken to delineate the type and degree of inhibition observed.

3.3   Binding and inhibition assay – saturation binding curves

Related saturation binding curves were generated by adding different amounts of rooibos extract (figs 9 – 11).

Using the amplitude of the type I difference spectrum as a measure of the degree of substrate binding, the correlation between the substrate concentration and the degree of binding was modelled using equation 1:

$$\Delta A = \frac{B_{max}[S]}{K_s + [S]} \tag{1}$$

Where:
ΔA is the amplitude of the binding spectrum;
B_{max} is the maximum substrate binding capacity of the enzyme;
K_s is the substrate dissociation constant;

In the presence of inhibitor, the values of the parameters B_{max} and K_s are altered:

$$K_s^{app} = \left(1 + \frac{[I]}{K_{si}}\right) K_s \qquad (2)$$

Where:
$[I]$ is the concentration of inhibitor;
K_{si} is the competitive binding inhibition constant;
K_s^{app} is the apparent substrate dissociation constant;

$$B_{max}^{app} = \frac{B_{max}}{1 + \frac{[I]}{K_i}} \qquad (3)$$

Where:
K_i is the noncompetitive binding inhibition constant;
B_{max}^{app} is the apparent substrate dissociation constant;

Substitution of equations 2 and 3 into equation 1 yields the following relationship:

$$\Delta A = \frac{B_{max}^{app}[S]}{K_s^{app} + [S]} \qquad (4)$$

For each extract, column fraction or phenolic compound under investigation, saturation substrate binding curves were generated at a number of different concentrations. Equations 2 and 3 were fitted separately to the inhibition data, weighted using the reciprocal of the asymptotic standard error calculated for each parameter.

Although binding inhibition studies cannot measure true noncompetitive inhibition, the term "noncompetitive inhibition" is used here to denote reduction in total binding capacity under conditions of substrate saturation. This can either be due to strong (essentially irreversible) inhibitor binding, or due to irreversible changes to the enzyme conformation.

The competitive inhibition constant can be seen as the concentration of inhibitor required to double the apparent substrate dissociation constant, and the noncompetitive inhibition constant as the concentration of inhibitor required to halve the maximum binding capacity. All inhibition constants are therefore reported in units of mg of dried plant material per mL microsomal suspension, except in the case of the column fractions, where the units are mg of lyophilized column effluent per mL.

The asymptotic standard errors of the half-maximal and maximal binding parameters determined from the fit of the measured saturation data to equation 4 were used as weights in the fit of the competitive and noncompetitive binding equations. To visualise the type of inhibition in each case, and to confirm the relevance of the inhibition model chosen, the resulting parameters were substituted into the equation for the hyperbolic curve, and plotted together with the substrate binding data on double-reciprocal plots. The calculated inhibition parameters (table 2) yielded sets of lines

which correlated well with the binding inhibition data when plotted in this linearized form.

The influence of phenolic components of rooibos, aspalathin, nothofagin, 3,4 dihydroxybenzoic acid, caffeic acid, luteolin, vitexin, quercetin 3-β-D-glucoside, quercetin dihydrate, ferulic acid, rutin hydrate, syringic acid, vanillic acid and p-coumaric acid, (*9*), on P4 binding to adrenal microsomal P450 was investigated.

Even at 100 μg/L, a much higher concentration than that at which they are present in rooibos tea (*9*), none of the polyphenols tested showed any effect on substrate binding.

3.4 Discussion

Plants express a variety of radical-scavenging species, of which the most well-known is ascorbic acid (*31*), a highly water-soluble vitamin. The powerful radical-scavenging effects of rooibos, however, are generally ascribed to its polyphenol content, especially the dihydrochalcone aspalathin (*4*). In this study, this was confirmed by the fact that the most hydrophilic fractions of rooibos, which had negligible polyphenol content, also had very little radical scavenging activity, and that the radical scavenging activity of the fractions increased in a linear fashion with their polyphenol content. It has been shown using aqueous extracts that the fermentation process reduces the antioxidant activity of rooibos (*17*). This study confirms that the difference between unfermented and fermented rooibos leads to different antioxidant and polyphenol profiles after HIC fractionation of the aqueous extracts.

Together with its radical-scavenging activity, the antimutagenic and anticarcinogenic activity of rooibos has been unambiguously demonstrated (*7, 32*). However, the stress-relieving effects of rooibos, well known in South African oral tradition, are not satisfactorily explained by its antioxidant or polyphenol capacity alone.

All rooibos extracts tested showed significant, dose-dependent mixed inhibition of P4 binding to the adrenal P450 enzymes, and both column fractions showed significant dose-dependent inhibition with respect to the non-competitive inhibition constant. The fact that the fermentation process, while strongly influencing the polyphenol profile of rooibos, did not have a marked effect on the degree of inhibition shown by the extracts further supports our hypothesis that components other than the polyphenols are responsible for the observed results. The chloroform extracts of both unfermented and fermented rooibos, containing the more hydrophobic components, showed stronger competitive inhibition than the corresponding methanol extracts, and the methanol extracts conversely showed stronger non-competitive effects than the corresponding chloroform extracts. This is to be expected, considering the hydrophobic nature of the natural substrates of the enzymes involved.

Although neither of the column fractions showed competitive inhibition of P4 binding, both showed strong non-competitive inhibition, with the more hydrophobic fraction showing stronger inhibition.

As both CYP17 and CYP21 show a type I binding spectrum upon binding of P4, *ex vivo* substrate binding experiments cannot identify which isoform is inhibited by rooibos extracts. However, if CYP21 were preferentially inhibited, this would decrease the flux towards the mineralocorticoids and glucocorticoids and increase the flux towards the androgen precursors. A reduction in glucocorticoid levels could conceivably mitigate the effects of an inappropriate acute stress response. This finding, which needs to be confirmed by enzyme kinetic and in vivo studies, is in line with the reputed health benefits of rooibos. Moreover, fractions of aqueous extracts, as well as fermented and unfermented rooibos extracts exhibit activity towards these enzymes, which demonstrates the relevance of these results to aqueous infusions – the normal mode of consumption of rooibos.

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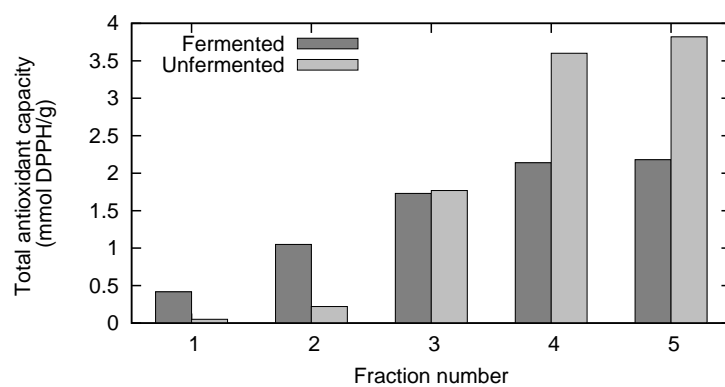
Figure 1: Comparison of the antioxidant capacity of HIC fractions of fermented and unfermented rooibos. All assays performed in singlicate. Fraction 1: most hydrophilic – Fraction 5: most hydrophobic
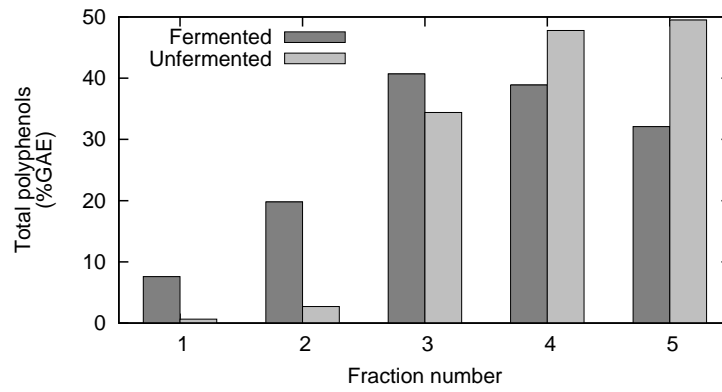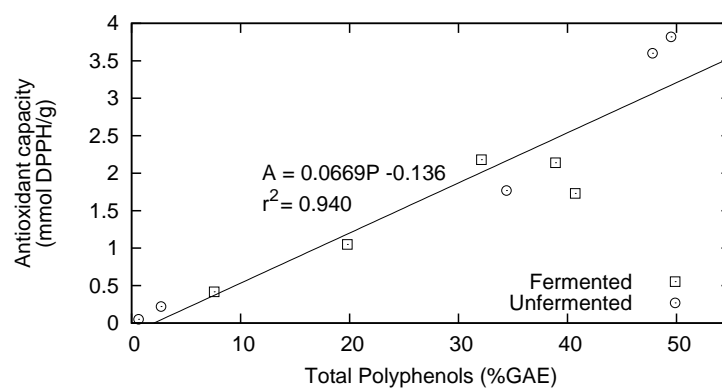
Figure 2: Comparison of the polyphenol concentrations of HIC fractions of fermented and unfermented rooibos. All assays performed in singlicate. Fraction 1: most hydrophilic – Fraction 5: most hydrophobic

Figure 3: Relationship between polyphenol concentration and antioxidant capacity of HIC fractions of fermented and unfermented rooibos, correlation coefficient $r^2 = 0.940$ for the combined data set.
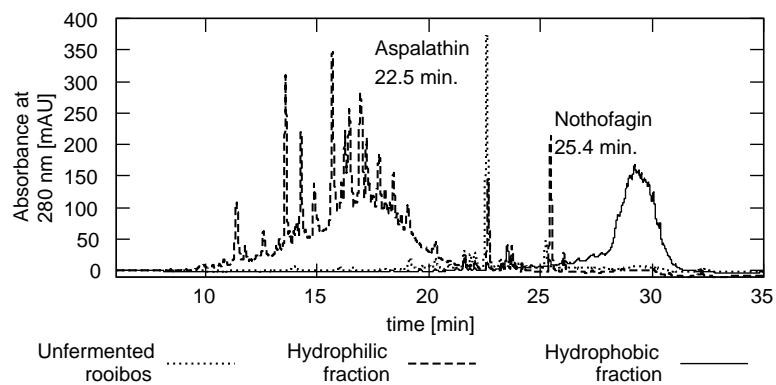
Figure 4: RP-HPLC of unfermented rooibos and hydrophobic and hydrophilic unfermented rooibos fractions. Zorbax SB-C18 column, particle size 3.5 μm, 3.0×150 mm.
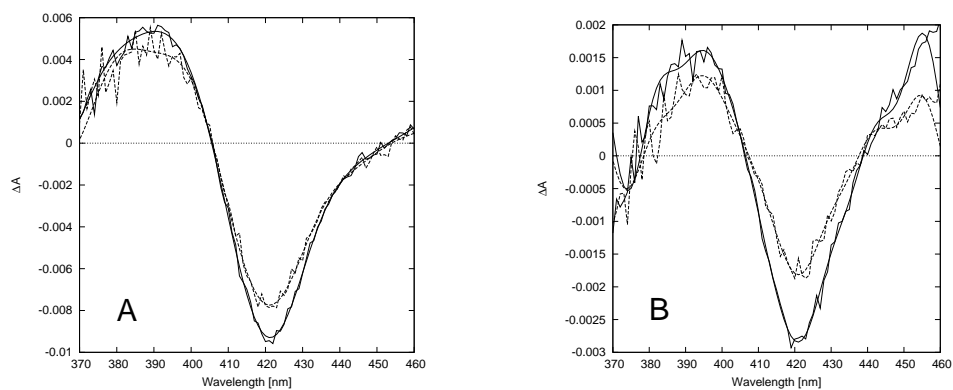
Figure 5: Inhibition of substrate-induced type I difference spectra in ovine adrenal microsomes, [cytochrome P450] = 0.3 μM, [P4] = 3.2 μM, by (A) unfermented and (B) fermented rooibos MeOH extracts (final concentration in terms of dried plant material = 1.0 mg/mL). Substrate-induced difference spectra in the absence (—) and in the presence (– –) of extract recorded between 370 and 460 nm; unprocessed curves shown together with Fourier smoothed data.
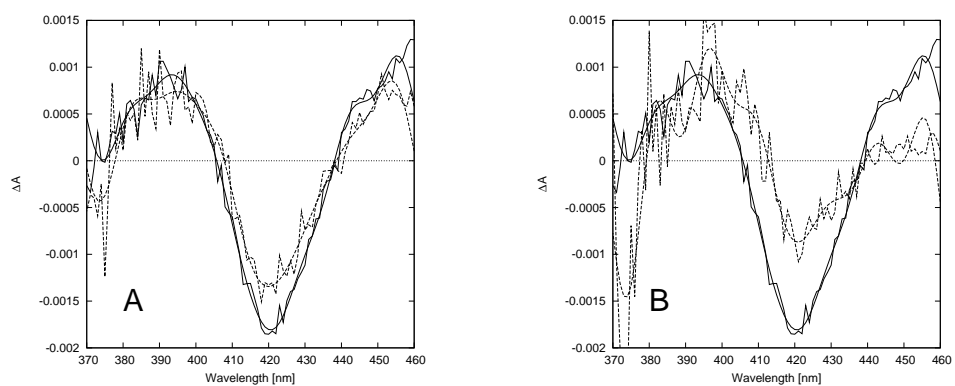
Figure 6: Inhibition of substrate-induced type I difference spectra in ovine adrenal microsomes, [cytochrome P450] = 0.3 μM; [P4] = 3.2 μM, by HIC column fractions. Substrate-induced difference spectra in the absence (—) and in the presence (– –) of extract, A: 0.29 mg hydrophilic fraction/mL and B: 0.145 mg hydrophobic fraction/mL; concentrations in terms of mass of lyophilized eluate; unprocessed curves shown together with Fourier smoothed data.
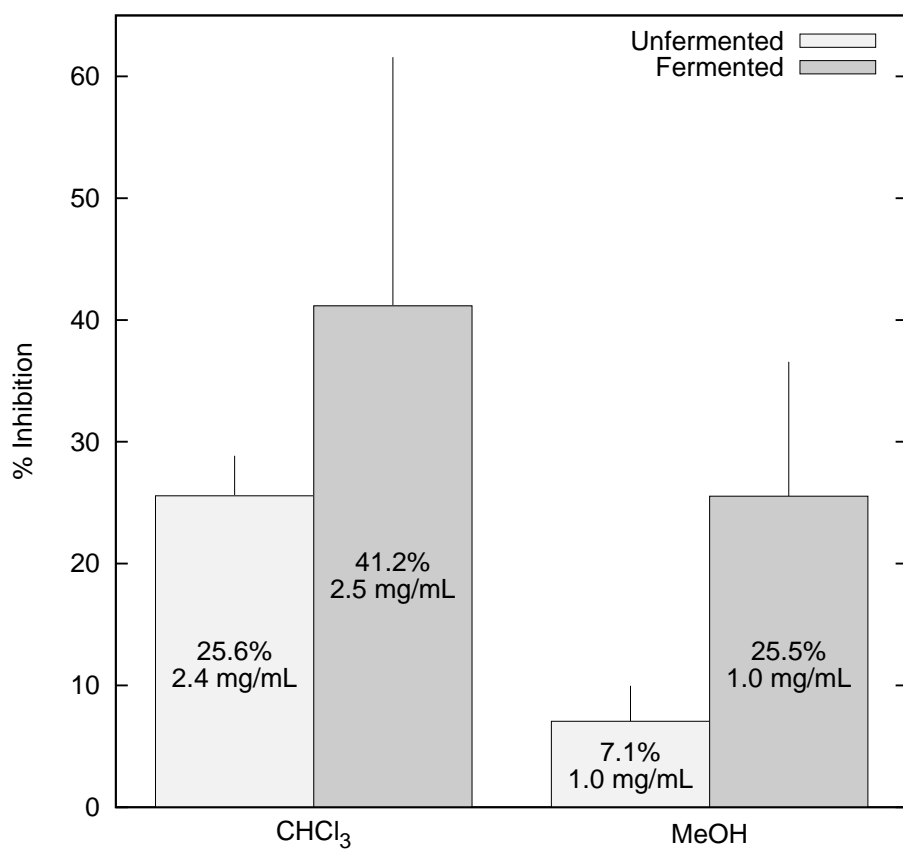
Figure 7: Percentage inhibition of P4 (3.2 μM) binding to adrenal microsomal P450 enzymes ([Cytochrome P450] = 0.3 μM) by unfermented and fermented rooibos chloroform extracts (2.4 and 2.5 mg/mL respectively) and by unfermented and fermented rooibos methanol extracts (final concentration 1.0 mg/mL). Analyses in duplicate; results are presented as the correlated value, error bars represent asymptotic standard error.
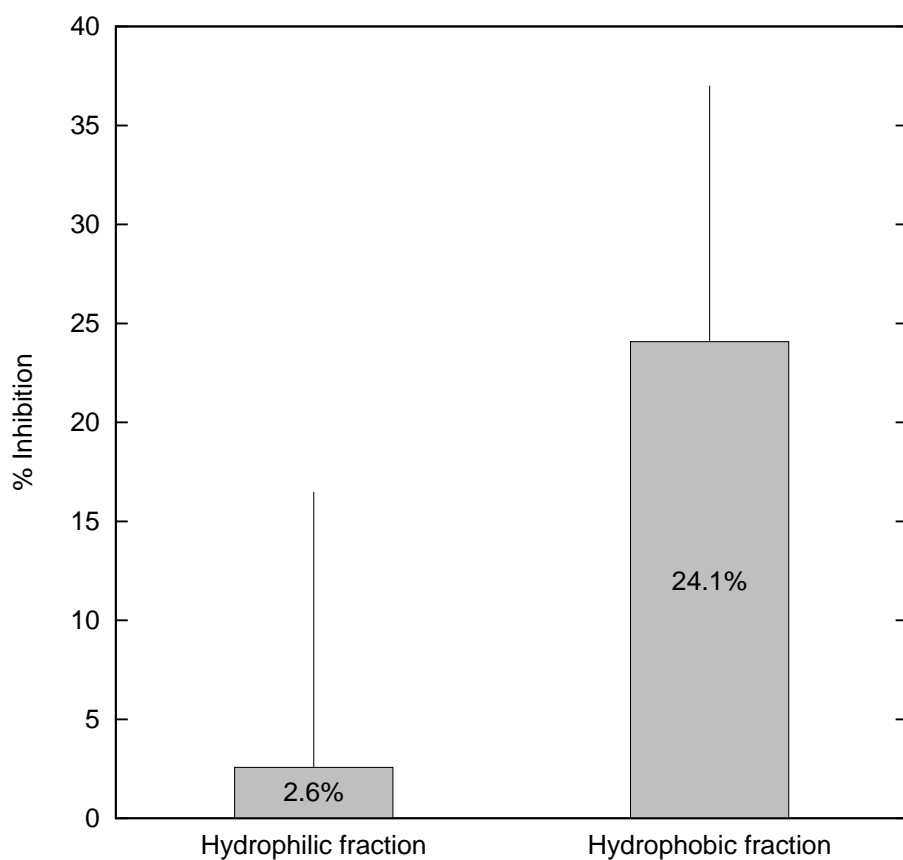
Figure 8: Percentage inhibition of P4 (3.2 μM) binding to adrenal microsomal P450 enzymes ([cytochrome P450] = 0.3 μM, by HIC hydrophilic and hydrophobic column fractions (final concentration 0.15 mg/mL). Analyses in duplicate; results are presented as the correlated value, error bars represent asymptotic standard error.
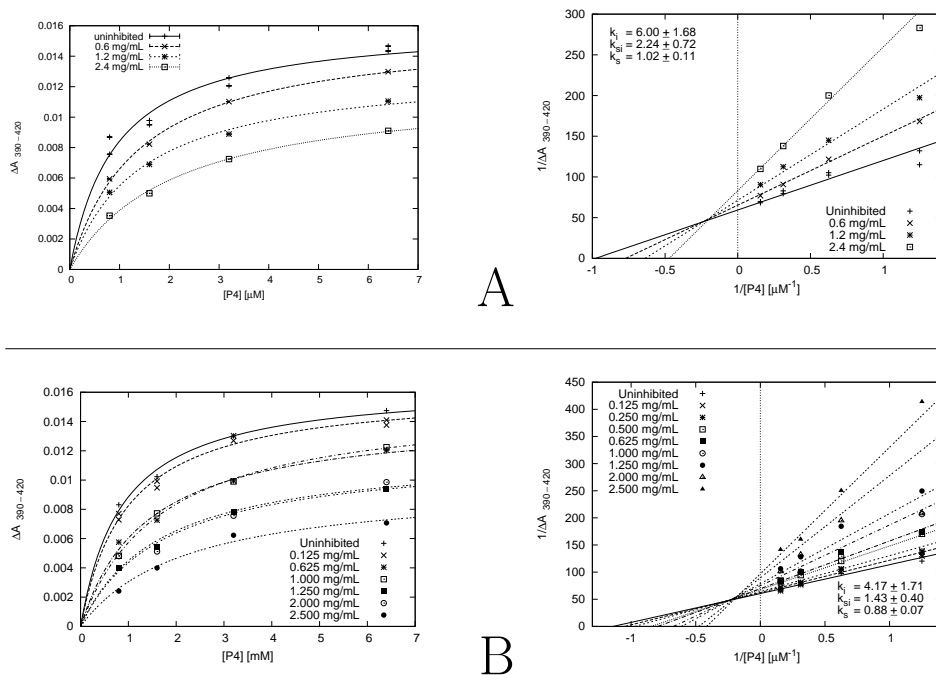
Figure 9: Hyperbolic saturation binding curves and double-reciprocal plots of the inhibitory effect of unfermented rooibos extracts on P4 binding to cytochrome P450; [P4] = 0.8 – 6.4 μM, [P450] = 0.3 μM. **A**: CHCl$_3$ extracts (final concentration, 0.6 – 4.8 mg/mL); **B**: MeOH extracts (final concentration, 0.125 – 12.50 mg/mL); analyses in duplicate.
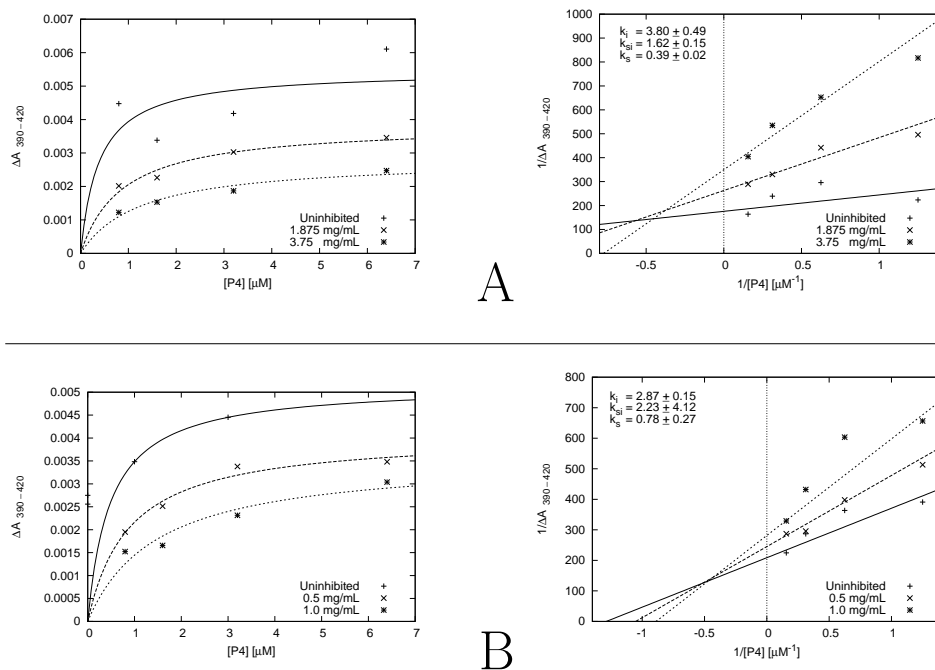
Figure 10: Hyperbolic saturation binding curves and double-reciprocal plots of the inhibitory effect of fermented rooibos extracts on P4 binding to cytochrome P450. [P4] = 0.8 μM – 6.4 μM, [P450] = 0.3 μM. **A**: CHCl$_3$ extracts (final concentration, 0.62 – 3.75 mg/mL; **B**: MeOH extracts (final concentration, 0.128 – 4.0 mg/mL); analyses in duplicate.
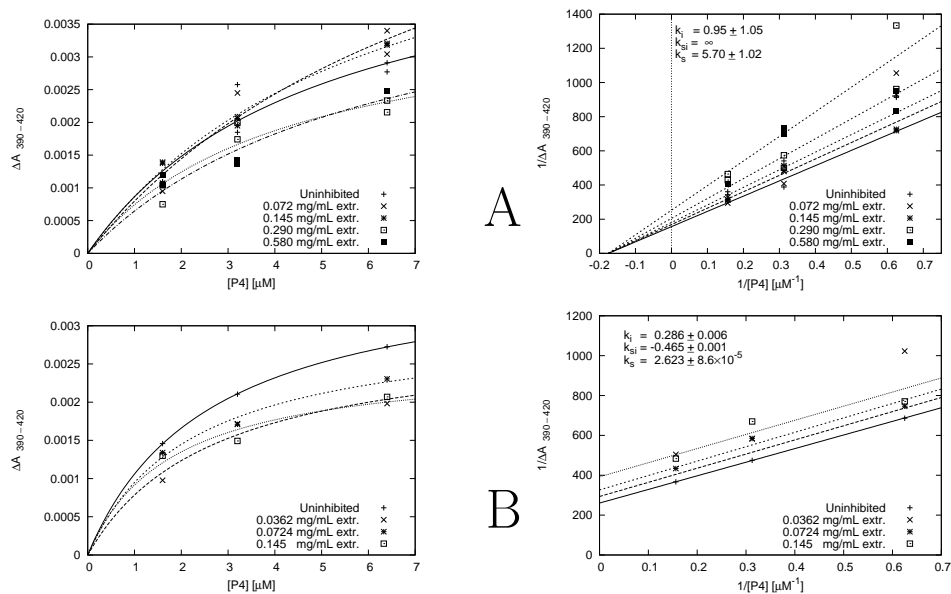
Figure 11: Hyperbolic saturation binding curves and double-reciprocal plots of the inhibitory effect of unfermented rooibos HIC column fractions on P4 binding to cytochrome P450; [P4] = 0.8 μM – 6.4 μM, [P450] = 0.3 μM **A**: hydrophilic column fraction (final concentration 0.072 – 0.58 mg/mL); **B**: hydrophobic column fraction (final concentration, 0.036 – 0.145 mg/mL; analyses in duplicate.

Table 1: Quantitative analysis of polyphenols in rooibos samples

Compound name	Unfermented rooibos	Unfermented HIC fraction [1]	Fermented rooibos	Fermented HIC fraction[1]
Aspalathin	10%	65%	0.53%	1.0%
Nothofagin	1.3%	0.60%	0.11%	0.06%
Isovitexin	0.18%	ND [2]	ND	0.11%
Luteolin	ND	ND	0.084%	ND
Vitexin	0.43%	1.1%	0.46%	0.42%
Quercetin-3-β-D-glycoside	0.16%	ND	0.13%	0.08%
Quercetin dihydrate	ND	ND	0.075%	ND
Rutin Hydrate	0.60%	0.94%	ND	0.59%
3,4 DHBA	ND	ND	0.22%	0.034%
Caffeic acid	0.022%	ND	0.04%	ND
Ferulic acid	ND	ND	0.17%	ND
p-Coumaric acid	ND	ND	ND	ND
Vanillic acid	0.023%	ND	0.09%	ND
Syringic acid	0.64%	ND	ND	0.11%

[1]fraction 3 in figure 1
[2]not detected

Table 2: Inhibition parameters determined for various rooibos preparations with respect to the binding of P4 to ovine adrenal P450. All parameters are reported as mg/mL plant material, except in the case of the column fractions, which are given as mg/mL lyophilized powder.

		K_{si}	K_i
Unfermented rooibos	Chloroform	2.24 ± 0.72	6.00 ± 1.68
	Methanol	1.43 ± 0.40	4.17 ± 1.71
Fermented rooibos	Chloroform	1.62 ± 0.15	3.80 ± 0.49
	Methanol	2.23 ± 4.12	2.87 ± 0.15
Column fraction	Hydrophilic [1]	ND	0.95 ± 1.05
	Hydrophobic [2]	ND	0.286 ± 0.006

[1]fraction 1 in figure 1

[2]fraction 5 in figure 1