BIOLOGICAL PROPERTIES OF SELECTED FLAVONOIDS OF ROOIBOS

(*Aspalathus linearis*)

Petra W Snijman

Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Chemistry

at the University of the Western Cape

Study Leader:	Prof IR Green
Co-study Leaders:	Prof E Joubert
	Prof WCA Gelderblom

June 2007

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.

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Petra Wilhelmina Snijman Date

ABSTRACT

Bioactivity-guided fractionation was used to identify the most potent antioxidant and antimutagenic fractions contained in the methanol extract of unfermented rooibos (*Aspalathus linearis*), as well as the bioactive principles for the most potent antioxidant fractions. The different extracts and fractions were screened using *Salmonella typhimurium* tester strain TA98 and metabolically activated 2-acetoaminofluorene (2-AAF) to evaluate antimutagenic potential, while the antioxidant potency was assessed by two different *in vitro* assays, i.e. the inhibition of Fe(II) induced microsomal lipid peroxidation and the scavenging of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. The most polar XAD fraction displayed the most protection against 2-AAF induced mutagenesis in TA98. Successive fractionation of the two XAD fractions most active in the $ABTS^{\bullet+}$ assay led to both aspalathin and nothofagin being isolated for the first time to a purity of >95%.

Thirteen flavonoids of rooibos were also compared in the two antioxidant assays in addition to a metal chelating assay in order to derive a possible comparative structure-activity profile between the dihydrochalcones aspalathin and nothofagin, their flavone analogues orientin and isoorientin (from the precursor aspalathin) and vitexin and isovitexin from nothofagin, the flavone aglycones luteolin and chrysoeriol as well as four flavonols from rooibos, i.e. quercetin, isoquercitrin (quercetin-3-*O*-glucoside), hyperoside (quercetin-3-*O*-galactoside) and rutin (quercetin-3-*O*-rutinoside). The flavanol (+)-catechin was also included while

epigallocatechin gallate (EGCG), the major active principle from *Camellia sinensis* was used as benchmark. Aspalathin, the dihydrochalcone unique to rooibos, was a very efficient inhibitor of lipid peroxidation and scavenger of $ABTS^{\bullet+}$, while nothofagin was equipotent to aspalathin in the $ABTS^{\bullet+}$ assay, but had the lowest inhibitory effect of all the flavonoids tested in the lipid peroxidation assay. Aspalathin was shown to be the major contributor to the antioxidant activity of unfermented rooibos in quantity and potency.

The same thirteen flavonoids of rooibos were also investigated in the *Salmonella typhimurium* mutagenicity assay, again using EGCG as benchmark. Strains TA98 and TA100 utilizing 2-AAF and aflatoxin B_1 (AFB_1) respectively, were used. Neither aspalathin nor nothofagin displayed potent antimutagenic properties against both the mutagens evaluated while luteolin was the most effective antimutagen. The antimutagenic behaviour of rooibos could not be solely attributed to any of these monomeric flavonoid constituents. No clear and direct link between the antioxidant and antimutagenic properties of the major rooibos flavonoids could be conclusively be established in the present studies.

AIMS AND OBJECTIVES

1. To investigate whether the same flavonoids are responsible for both the potent antioxidant and antimutagenic properties of unfermented rooibos.
2. To compare the activity of thirteen of the major flavonoids of unfermented rooibos in two antioxidant assays.
3. To compare the antimutagenic activity of the same thirteen flavonoids of unfermented rooibos in two different *Salmonella typhimurium* strains using two different metabolically activated mutagens.

ACKNOWLEDGEMENTS

This thesis is the result of many years of work and it realized thanks to the support of, amongst others, the following people and institutions:

I would first-off like to express my deep and sincere gratitude towards Prof Ivan Green, my study leader, for being a true mentor to me. Thank you for your encouragement, understanding and personal guidance, always given in a positive, gentle and friendly manner. I have learnt so much from you.

I would like to thank Dr Wentzel Gelderblom of the Medical Research Council, my former project leader, for defining this project and allocating me to this fascinating work. Thank you for the technical skills you taught me. Thank you for your patience with me and teaching me to have patience with columns and other laboratory routines.

I owe a sincere thanks to Dr Lizette Joubert, not only for the weeks of training I spent at ARC Infruitec-Nietvoorbij and sharing her insights and expertise with me throughout this project, but also for always being ready to enthusiastically think up a new angle to an argument or another possible solution to a problem. I benefited much from her about rooibos, its flavonoids and analytical chemistry.

I am also endebted to the following people:

Prof Kayoko Shimoi of the University of Shizuoka, Japan, for her valuable contribution in optimising the lipid peroxidation assay;

Ms Sonja Swanevelder of the Medical Research Council and Dr Martin Kidd of the Department of Statistics at the University of Stellenbosch for statistical analyses;

Dalene de Beer of ARC Infruitec-Nietvoorbij for training and assistance in HPLC analysis and the $ABTS^{\bullet+}$ assay;

Shamiel Joseph and Dr Jeanine Marnewick for their assistance in the *Salmonella typhimurium* assay;

Dr BN Ames and colleagues of the University of California in Berkeley for kindly supplying the *Salmonella typhimurium* tester strains;

Dr Ann Louw and her students Ciko Mfenyana and Steven Robertson of the Biochemistry Department at the University of Stellenbosch for valuable assistance with the Prism computer programme;

Dr Thinus van der Merwe (then of the University of Stellenbosch) for LC-MS spectra of aspalathin and nothofagin;

Dr Robert Vleggaar of the University of Pretoria for NMR-spectra of aspalathin;

The Medical Research Council, Rooibos Tea Forum and THRIP for financial support;

Mercia Mottie, Faculty Officer at the University of the Western Cape for always being friendly and helpful with registration and any administrative enquiry or problem;

My former colleagues at the Medical Research Council where the experimental work was done, for their support, advice and inputs, and John for always having clean glassware.

To my present colleagues at the University of Stellenbosch I have such appreciation and gratefulness:

Prof Jan Dillen and Dr Catharine Esterhuysen: thank you for your support and flexibility in granting me leave from work;

Japie, Glen and Jessie: thank you for your interest shown and for being prepared to fill in for me;

Moebarick and Johannes for your assistance and support;

Maryna, thank you for your encouragement and perspectives, and for reminding me about the Bigger Picture!

And last but by no means least: I want to thank my mother, sisters and friends for their interest and support throughout this time. Yes, it is finally done!

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Every chapter of this thesis has been written as an individual entity that could form the basis of a journal manuscript. Repetition between chapters has therefore been unavoidable.

Key to abbreviations used in thesis

$^{\bullet}OH$	:	hydroxyl radical
2-AAF	:	2-acetylaminofluorene
$ABTS^{\bullet}$	:	2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical
AFB_1	:	aflatoxin B_1
BHP	:	*tert*-butylhydroperoxide
BHT	:	butylated hydroxytoluene
CHP	:	cumene hydrogen peroxide
COMT	:	catechol-*O*-methyl transferase
DMSO	:	dimethyl sulfoxide
$DPPH^{\bullet}$	:	2,2'-diphenyl-1-picrylhydrazyl radical
EDTA	:	ethylenediaminetetraacetic acid
EGCG	:	epigallocatechin gallate, a green tea flavonoid used as reference in this study
Fl-OH	:	flavonoid
$Fl\text{-}O^{\bullet}$	:	aroxyl radical
HPLC	:	High Performance Liquid Chromatography
IQ	:	2-amino-3-methylimidazo[4,5-*f*]quinoline
$L^{\bullet}$	:	alkyl radical
LH	:	polyunsaturated fatty acid
$LO^{\bullet}$	:	lipid alkoxyl radical
$LOO^{\bullet}$	:	lipid peroxyl radical
LOOH	:	lipid hydroperoxide
PhIP	:	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
$RO_2^{\bullet}$	:	peroxyl radical
S9-mix	:	Aroclor-induced rat liver homogenate fraction used to metabolic activate mutagens in *Salmonella* mutagenicity assay
$O_2^{\bullet -}$	:	superoxide radical
TBA	:	thiobarbituric acid
TBARS	:	thiobarbituric acid reactive substances
TCA	:	trichloroacetic acid
TLC	:	Thin Layer Chromatography

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CHAPTER 1

INTRODUCTION

Rooibos tea is a traditional beverage of the Khoi-descended people of the Cedarberg region in the Cape. The plant is endemic to South Africa and its natural distribution area is the Cedarberg area (Figure 1.1) that includes Clanwilliam, Nieuwoudtville and Piketberg, all of which have the deep, acidic, sandy soils in which rooibos grows. The herbal drink prepared from *Aspalathus linearis* has already been reported as early as 1772 by Carl Thurnberg, but trading in rooibos started in 1904 when the Khoi started selling rooibos to Benjamin Ginsberg. The tea was harvested from wild rooibos until the 1930's after which rooibos was developed into a crop plant. One of the key players in the commercialisation of rooibos was the well known and South African poet, cook, doctor and botanist Louis Leipoldt. He and another doctor from Clanwilliam, P le Fras Nortier, recognised the medicinal as well as commercial potential of rooibos and started cultivating the shrubs on Nortier's farm. Since that time rooibos plantations are a common sight in that region (Figure 1.2). The Rooibos Tea Control Board was established in 1954 to stabilise producer prices through structured marketing and quality control. This Board was later transformed into a private company in 1997 (Van Wyk and Gericke, 2003; Dugmore, 2004; Joubert and Schulz, 2006).

Today rooibos is very popular as a health beverage, prepared and used much in the same way as black tea. It has in latter years gained popularity as an excellent iced tea beverage. Rooibos's antispasmodic property makes it a calming drink for

Figure 1.1. Rooibos is endemic to the Cedarberg region, indicated in yellow, of South Africa.
Map from Medicinal Plants of South Africa, 2002

Figure 1.2. A typical rooibos (*Aspalathus linearis*) plantation
Photograph courtesy of Dr E Joubert

babies. It is also used as an ingredient in cosmetics, in slimming products, as a flavouring agent in cakes, cooking and cocktails (Van Wyk et al., 2002; Van Wyk and Gericke, 2003).

Rooibos contains a unique dihydrochalcone, aspalathin, as well as its 3-dehydroxy analogue, nothofagin, that until now have only been found in *Nothofagus fusca* (Hillis and Inoue, 1987; Joubert, 1996). These compounds, together with isoorientin, orientin and rutin, are the most commonly found in fermented rooibos while aspalathin and nothofagin are present in higher concentrations in the unfermented rooibos than the fermented version (Joubert, 1996; Bramati et al., 2002; Bramati et al., 2003; Joubert and Schulz, 2006). In addition unfermented rooibos has a higher antioxidant capacity than fermented rooibos (Von Gadow et al., 1997; Standley et al., 2001). Standley et al. (2001) also found that the process of fermentation reduced the antioxidant and antimutagenic activity of a rooibos extract. It has already been shown that aspalathin exerts a strong scavenging ability against $DPPH^{\bullet}$ and $O_2^{\bullet -}$ (Joubert et al., 2004). Unfermented rooibos extracts with high levels of aspalathin are increasingly in demand by international cosmetic and functional food markets (Joubert and Schulz, 2006).

Yen and Chen (1995) investigated the antioxidant activity and antimutagenicity of Japanese tea extracts at various stages of fermentation and found the degree of correlation depended on the antioxidant assay used, mutagen utilized and the state of fermentation of the extract.

This lead to the following questions regarding the antioxidant and antimutagenic properties of rooibos being posed:

1. Could these two properties in some way be linked?
2. Would aspalathin, known as a potent antioxidant and a major contributor to the antioxidant properties of rooibos, also be a major contributor to the antimutagenic properties of rooibos?
3. Is nothofagin biologically as important as aspalathin?

If these two dihydrochalcones were found to be the more important bioactive compounds of rooibos, then clearly the fermentation process would impair the biological activity of rooibos and thus the necessary adjustments in the processing would have to be made to minimize the loss in activity and thus provide for a better product.

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CHAPTER 2

LITERATURE REVIEW

Background of industry

Rooibos (*Aspalathus linearis* Fabaceae) is a traditional South African beverage and is one of only a few indigenous plants that have become an important commercial crop (Van Wyk and Gericke, 2003). The stems of this fynbos legume are mostly cut with a sickle and tied into bundles. The traditional processing involves comminution and bruising after which the 'fermenting' takes place. During 'fermentation' the fine segments of rooibos are left in heaps in the sun to 'sweat' or 'ferment' during which time the characteristic red-brown colour and sweetish flavour develops (Joubert, 1996; Joubert and Schulz, 2006). This 'fermentation' process is actually a process of chemical oxidation during which the flavonoids of the plant are enzymatically oxidized. The tea is then spread out to dry in the sun (Joubert, 1996; Van Wyk and Gericke, 2003). Rooibos is popular as a health beverage, contains no harmful stimulants like caffeine and is low in tannins (Blommaert and Steenkamp, 1978; Ferreira et al., 1995). Domestic consumption of rooibos has increased fairly steadily over the years, 3600 tons being the long-term average local supply, accounting for 70% to 75% of annual production (Arendse, 2001). Major importers include Germany, Japan, the Netherlands, England, Malaysia, South Korea, Poland, China and the United States. In 1999, about 29 percent of South Africa's total rooibos sales were exported to 31 countries (Erickson, 2003). In 2001, a total of 3889 tons of rooibos was exported which increased to 6312 tons in 2003. The most

important importer of that year was Germany who imported 4661 tons (figures supplied by Perishable Products Export Control Board).

The international market for unfermented or 'green' rooibos has also developed over the last few years. The nutraceutical and cosmetic industries prefer extracts from unfermented rooibos since this provides the active principle, aspalathin, in much higher concentrations (Joubert and Schulz, 2006). Aspalathin occurs in fairly large quantities in both fermented and unfermented rooibos and is a powerful antioxidant (Joubert, 1996; Bramati et al., 2002; 2003; Joubert et al., 2004).

Biosynthesis of Flavonoids

Flavonoids are one of the largest groups of naturally occurring phenols. Smith (1972, as quoted by Markham, 1982) estimated that about 2% of all carbon synthesized by plants, equalling about 1×10^9 tons per annum is converted into flavonoids of closely related compounds. In plants, flavonoid aglycones contain fifteen carbon atoms that are arranged in a C_6-C_3-C_6 configuration, comprising two aromatic rings A and B linked by a three-carbon unit which may or may not form part of a third ring described as ring C. For convenience the rings are labelled A, B and C and numbered with ordinary numerals on the A-and C-rings and primed numerals for the B-ring. This numbering system differs for the chalcones (Figure 2.1) (Harborne and Baxter, 1999).

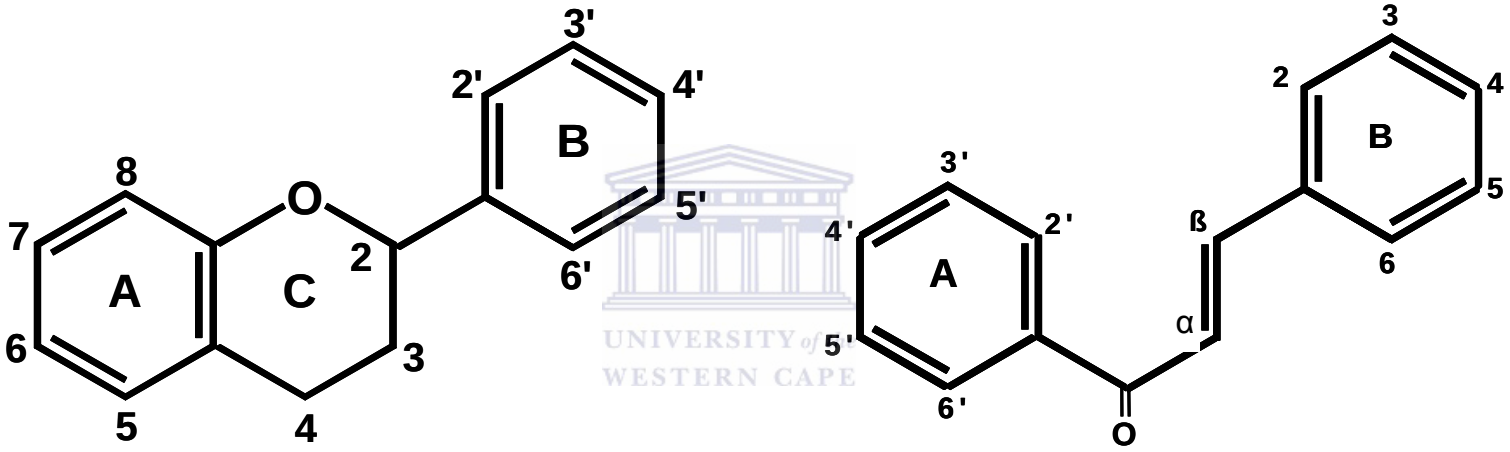


Figure 2.1. The general structure of flavonoids (left) and chalcones (right), after Harborne and Baxter (1999)

Besides their contribution to plant colour, flavonoids have a variety of other roles in plants such as protection against UV-B radiation and being eaten by animals, signalling for nitrogen fixation and pollen reception as well as being responsible for attractive colours, odours and flavours that will develop during ripening to advertise the readiness of the fruit to be eaten and subsequent seed dispersal. Many flavonoids are pharmacologically active and exhibit a wide range of biological properties, including antimicrobial, insecticidal, fungicidal, antiinflammatory, vasodilatory, anticancer and oestrogenic. Flavonoids occur widely in fruits and vegetables and it has been estimated that at least one gram of mixed flavonoids expressed as glycosides is taken in as part of a well balanced daily diet. However, in a Dutch national survey that came to be known as the Zutphen Elderly Study, it was found that the average intake of mixed flavonoids was only 26 mg/day (Kühnau, 1976; Mabry and Ulubelen, 1980; Hertog et al., 1993; Middleton et al., 1993; Harborne and Baxter, 1999; Harborne, 2001).

Flavonoids fall into two major categories depending on whether the central heterocyclic pyran ring is saturated or not. When unsaturation is present in the pyran ring C as in anthocyanins, flavones and flavonols, the molecule adopts a planar conformation. On the other hand when ring C is saturated the resulting flavonoids viz., flavanones and flavans may have one or more chiral centres and generally adopt a conformation in which the two benzene rings at right angles. Optical activity may also be present in flavonoids due to the presence of glycosidic substituents and chiral centres on ring C (Harborne and Baxter, 1999).

Flavonoids are generally classified according to their biosynthetic origin, with some classes being intermediates in the biosynthetic process, like the chalcones and flavanones, while other classes like flavones and flavonols, are end-products (Figure 2.2). Additional enzymic catalyzed protocols leading to many of the end products include *O*-methylation, *O*-acylation and *O*-glycosylation; the latter referring to sugar moieties naturally associated with flavonoids in conjugated form (Harborne and Baxter, 1999).

Rooibos flavonoids

Rooibos contains a distinctive selection of structurally related flavonoids that render themselves ideally suited for comparative structure-activity studies (Figure 2.3). Two of the major flavonoids, i.e. aspalathin and nothofagin, belong to the dihydrochalcones, a relatively small group of flavonoids with a random distribution in about 28 families, notably Ericaceae (Harborne and Baxter, 1999). The best known dihydrochalcone is phloridzin from the skin of apples. Since described for the first time in 1965 by Koeppen and Roux (1965) after its isolation from *Aspalathus linearis*, aspalathin has not yet been reported as being isolated from any other source. The only other known source of nothofagin, the 3-dehydroxy analogue of aspalathin, is the *Nothofagus fusca* tree (Hillis and Inoue, 1967; Joubert, 1996). Koeppen (1962) was also the first person to isolate the flavone-*C*-glycoside, orientin, from *Aspalathus acuminatus* and described the interconvertible relationship of orientin with homo-orientin (isoorientin), and later the flavonols rutin and isoquercitrin (Koeppen et al. 1962) (See Figure 2.3 for the structures). Snykers and Salemi (1974) contributed to

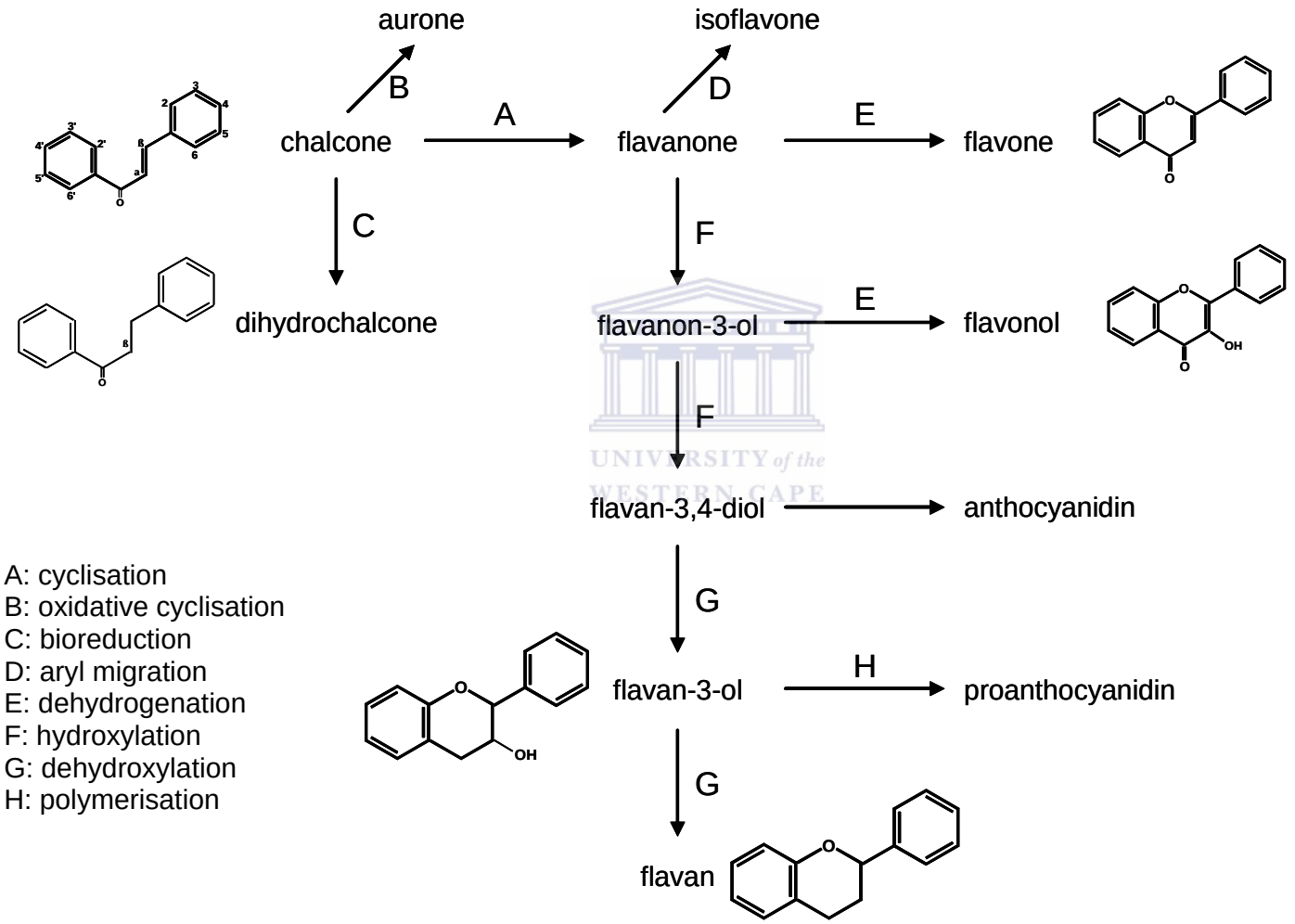
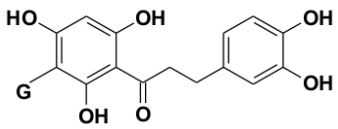
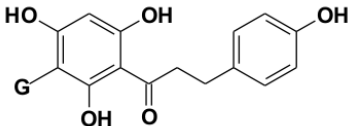


Figure 2.2. Biosynthetic pathways of some flavonoids (after Harborne and Baxter, 1999).

Dihydrochalcones

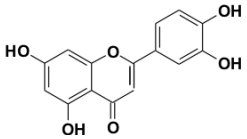


Aspalathin

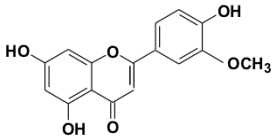


Nothofagin

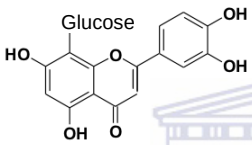
Flavones



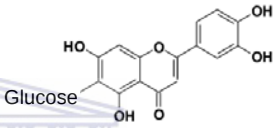
Luteolin



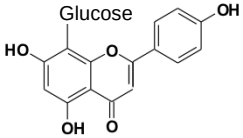
Chrysoeriol



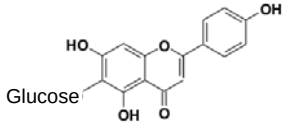
Orientin



Isoorientin

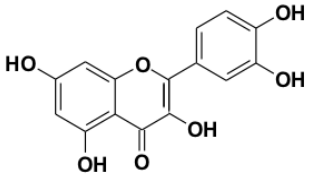


Vitexin

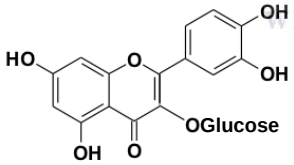


Isovitexin

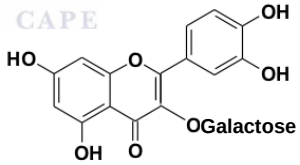
Flavonols



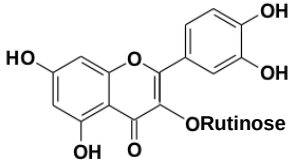
Quercetin



Isoquercitrin

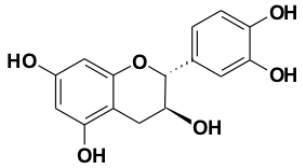


Hyperoside



Rutin

Flavanol



(+)-Catechin

Figure 2.3. Structures of the major flavonoids of rooibos (*Aspalathus linearis*)

this field of study with the isolation of the flavone luteolin and flavonol quercetin from commercially available rooibos in their search for the compound responsible for the antiallergic effect of rooibos. Rabe et al. (1994) were the first to identify the presence of isovitexin and vitexin, the 3′-dehydroxy analogues if isoorientin and orientin, respectively in rooibos. They also prepared an ether extract from an aqueous extract of commercial rooibos and found the following phenolic carboxylic acids present in this ether extract viz., 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxy-3-methoxy-benzoic acid (vanillic acid) as well as the hydroxycinnamic acids 4-coumaric acid, caffeic acid and ferulic acid. In addition chrysoeriol was identified for the first time as a constituent of the ether extract. The aqueous extract of rooibos was then extracted with ethyl acetate and this extract yielded 3,4,5-trihydroxycinnamic acid, a series of C-C linked *β*-D-glucopyranosides based on flavones, a dihydrochalcone and trihydroxycinnamic acid. The contribution of Ferreira et al. (1995) towards the phenolic profile of rooibos includes 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 4-hydroxy-3,5-dimethoxy cinnamic acid, luteolin-7-O-β-D-glucopyranoside, dihydro-orientin and dihydro-iso-orien-tin, 5,6-dihydroxy-6-*C*-β-D-glucopyranosylchromone, (+)-catechin, procyanidin B3, (+)-pinitol and a naturally occurring glycoside of phenylpyruvic acid. The flavonol quercetin-3-*O*-galactoside (hyperoside) as well as traces of the flavanones dihydroorientin and dihydro-isoorientin was identified by Bramati et al. (2002) in fermented rooibos. Kazuno et al. (2005) confirmed the presence of luteolin-7-*O*-glucopyranoside. In their search for phytoestrogens from rooibos, Shimamura et al. (2006) identified aspalalinin, a dihydrochalcone in which the A and B ring were linked via an ether bond. This suggests that there are currently

about 25 non-volatile known compounds contained in rooibos. Habu et al. (1985) identified 99 components in the steam distillate volatile oil fraction from fermented rooibos, which included an amazing 26 ketones, 19 aldehydes, 16 alcohols, 7 phenols, 4 acids and 3 ethers. A major component identified was guaiacol that is not present in *Camellia sinensis* teas. For the purpose of this study, however, the volatile components of rooibos were not considered. This study focussed specifically on the major water soluble flavonoids of rooibos, i.e. the two dihydrochalcones, aspalathin and nothofagin, the flavones luteolin, chrysoeriol, orientin, isoorientin, vitexin and isovitexin, the flavonols quercetin, hyperoside, isoquercitrin and rutin as well as the flavanol (+)-catechin (Figure 2.3).

Phenolic changes during fermentation

The exact extent to which the chemical profile of harvested rooibos is altered during fermentation is to the best of my knowledge not known as no study that involves the phenolic analysis of a single batch of rooibos, pre- and postfermentation, has appeared in the literature. It is however known that fermentation of rooibos alters the antioxidant activity and could either decrease or increase the mutagenic activity depending on the batch or mutagen (Joubert, 1996; Standley et al., 2001; Bramati et al., 2003; Van der Merwe et al., 2006). Bramati et al. (2003) did not analyse for nothofagin, but it was found in their HPLC-UV analysis that the major compounds (>1 mg/g dried mass), aspalathin, isoorientin, orientin and rutin, were more abundant in unfermented rooibos than in fermented rooibos. The phenolic profile of rooibos is most certainly altered by ‘fermentation’. This has been quantified by an HPLC comparative analysis of

unfermented and fermented rooibos, as well as total phenols (41.0% vs 35.0%, respectively (Joubert, 1996; Standley et al., 2001). In the abstract of a publication that could not be obtained through the interlibrary services of the University of Stellenbosch, Schmandke (2005) stated the one gram of unfermented rooibos (soluble dry matter) contained 50 mg of aspalathin, 0.1 mg aglycone, 2 mg C-O-linked flavonoid glycosides and 7 mg C-C-linked flavonoid glycosides compared to the reduced values 1.2, 0.2, 1.7 and 2.4 mg/g respectively in fermented rooibos. It was further clamed that aspalathin is almost completely oxidised to dihydroorientin, that rutin is partly converted to quercetin and that orientin, isoorientin, vitexin and isovitexin are partially degraded as well (Schmandke, 2005). According to Bramati et al. (2003), the aspalathin content can drop from as much as 49.9 to 1.2 mg aspalathin/g dried leaf mass, though the average of Joubert and Schulz (2006) of 6.6 g aspalathin/100 g dry unfermented rooibos to 0.26 g/100 g dry fermented rooibos seems more realistic. The average drop in nothofagin content is from 0.67 to 0.12 g/100 g during fermentation. Koeppen and Roux (1966) showed that aspalathin is converted to 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin in an ethanolic solution. However, when Marais et al. (2000) mimicked the 'fermentation' of rooibos, exposing aspalathin as substrate to heat and light, two diastereomeric flavanones, i.e. (*S*)- and (*R*)-eriodictyol-6-C-β-D-glucopyranoside were formed that may be further rearranged or oxidised to the corresponding flavones. The hypothesis that aspalathin could enzymatically be oxidised to the flavanones dihydro-orientin and dihydro-iso-orientin was strengthened by the presence of the glucopyranosyl chromone, a possible residue of the oxidative conversion of dihydro-iso-orientin (Ferreira et al., 1995).

Biosynthetically it still has not been shown that the dihydrochalcones are directly part of the flavone pathway. It is however chemically plausible that the dihydrochalcones oxidise to their corresponding flavones during the 'fermenting' conditions.

Bramati et al. (2002) identified two peaks in the mass spectra of fermented rooibos that could possibly be ascribed to dihydro-isoorientin and dihydro-orientin.

Health properties of rooibos

Many health properties have been linked to rooibos. These include antioxidant and antimutagenic properties (Von Gadow et al., 1997; Hitomi et al., 1999; Marnewick et al., 2000; Standley et al., 2001; Edenharder et al., 2002; Van der Merwe et al., 2006), estrogenic (Shimamura et al., 2006), antispasmodic (Snyckers and Galemi, 1974; Gilani et al., 2006), bronchidilatory (Khan and Gilani, 2006) and chemopreventive properties (Sasaki et al., 1993; Komatsu et al., 1994).

It is accepted that to a large extent it is the constituent flavonoids contained in those plants with a relatively high flavonoid content that give that plant its biological and pharmacological properties (Middleton et al., 1993). Since the focus of this study was centred on the antioxidant and antimutagenic properties of the flavonoids of rooibos this review will now focus on these two aspects.

Antioxidant activity of rooibos and its flavonoids

Unprocessed, semi-processed and processed rooibos was shown to have stronger 2,2'-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavengers than black and oolong teas, but weaker than green tea when compared on a mass equivalent basis (Von Gadow et al., 1997). Processing of rooibos decreases the antioxidant activity with regards to the superoxide and DPPH radical scavenging ability as well as the antimutagenic properties (Standley et al. 2001). The activity of several of the composite flavonoids of rooibos has been reported in various antioxidant assays. The radical scavenging activities of EGCG, catechin, quercetin, isoquercitrin, rutin and luteolin were compared using the 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) assay (Re et al., 1999; Lien et al., 1999, Plumb et al., 1999) whereas Okawa et al. (2001) evaluated the activities of isovitexin, quercetin and rutin. Joubert et al. (2004) included the rooibos flavonoids aspalathin, orientin, isoorientin, luteolin, isoquercitrin, (+)-catechin, rutin, vitexin and chrysoeriol in their assessment of the DPPH radical scavenging activities. Inhibition of lipid peroxidation in rat liver microsomes by epigallocatechin gallate (EGCG), catechin, luteolin, orientin, isoorientin, vitexin, rutin, hyperoside and quercetin has also been reported (Robak et al., 1988; Mora et al., 1990; Cos et al., 2001; Yang et al., 2001; Heijnen et al., 2002). Quercetin and EGCG are known to be potent antioxidants as measured in the ABTS radical cation scavenging assay and inhibitors of lipid peroxidation. Joubert et al. (2004) also concluded that the DPPH and superoxide anion radicals were most effectively scavenged by quercetin.

Antioxidant assays

General

Antioxidants perform many functions ranging from phytoprotectants in plants, to protecting lipids in food products, to *in vivo* antioxidant activity in animals and humans, including scavenging reactive oxygen species, acting as antagonists toward oxidative enzymes such as cyclooxygenases and influencing the expression of multiple genes (Finley, 2005). Huang et al. (2005) concluded that a dietary antioxidant can (sacrificially) scavenge reactive oxygen/nitrogen species to stop radical chain reactions, or it can inhibit the reactive oxidants from being formed in the first place (preventive). Non-enzymatic or dietary antioxidants can generally include radical chain inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors (Figure 2.4). Whereas autoxidation of a lifeless matter occurs by radical chain reactions, oxidation in a biological system is primarily mediated by a host of redox enzymes. Nonetheless may nonenzymatic lipid autoxidation by radical chain reaction still occur and lead to oxidative stress. Consequently biological antioxidants include enzymatic antioxidants (e.g. superoxide dismutase and catalase) and nonenzymatic antioxidants such as oxidative enzyme inhibitors like cyclooxygenase, antioxidant enzyme cofactors, reactive oxygen/nitrogen species scavengers and transition metal chelators (Figure 2.4).

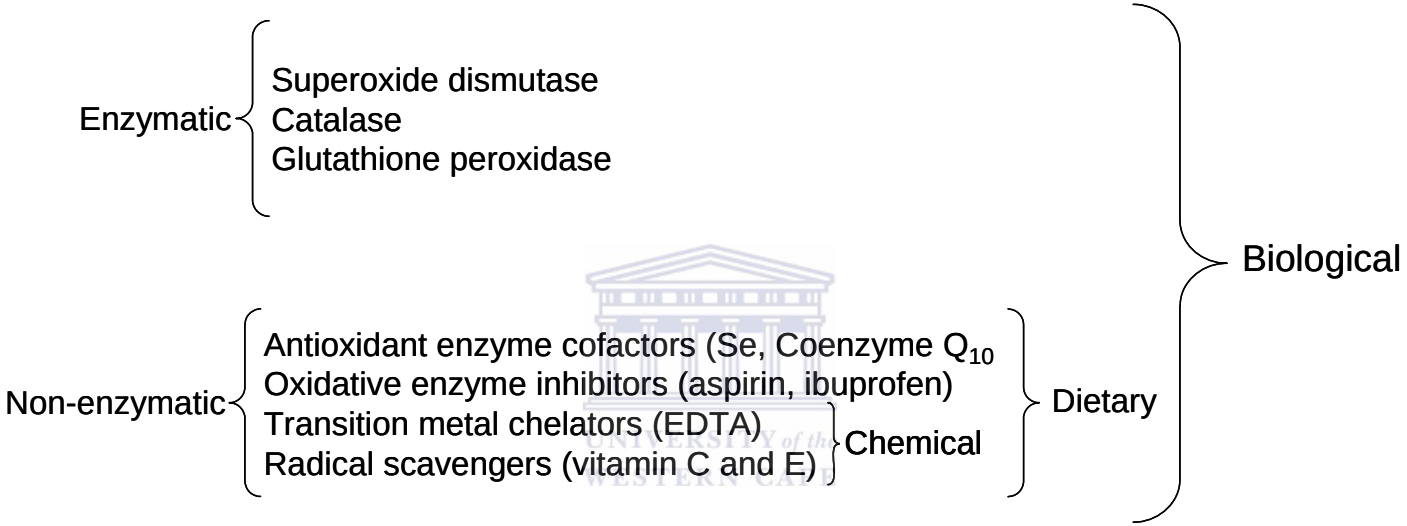


Figure 2.4. Scope of biological antioxidants (after Huang et al., 2005).

Halliwell (1990) defined biological antioxidants as 'molecules which, when present in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules'. A broader definition of an antioxidant for the context of this study, would be 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate' (Halliwell, 1990).

The antioxidant activity of flavonoids can be related to: i) the scavenging of free radicals since, due to their lower redox potential, they are able to reduce highly oxidising free radicals such as superoxide, peroxyl and hydroxyl (Torel et al., 1986; Husain, 1987; Robak, 1988; Bors et al., 1990); ii) chelating transition metals involved in free radical production (Morel et al., 1993; Pietta, 2000); and iii) inhibiting the enzymes from participating in free radical generation such as xanthine oxidase (Cotelle et al., 1996). This activity involves a chemical process which depends on the redox, partitioning, chelating, hydrogen-donating and radical scavenging properties of a compound (Bravo, 1998; Williamson et al., 1999; Re et al., 1999; Sugihara et al., 1999). Frankel and Meyer (2000) distinguished the following possible roles of phenolic compounds as antioxidants viz., preventors of oxidant formation, scavengers of activated oxidants in order to reduce active intermediates as well as to induce repair mechanisms. They recommended that specific products of oxidation be measured in relevant *in vitro* and *in vivo* biological systems. Cos et al. (2001) emphasized the importance of working with test solutions of potential plasma levels, i.e. a promising antioxidant compound should show a lipid peroxidation

inhibiting effect at micromolar level and further demonstrate a low cytotoxicity on growing cells.

An antioxidant can exert its effect by different mechanisms and/or functions and it is essential that when evaluating the antioxidant activity of a molecule the function that is being measured by the method employed must be clearly identified. The capacity of the antioxidants *in vivo* is determined not only by their reactivity towards the radical, but also by factors such as concentration, distribution, localisation, fate of the antioxidant-derived radical, interaction with other antioxidants and metabolism. Quite often it is not the antioxidant activity that is measured, but the actually the reactivity towards the radical (Niki and Noguchi, 2000). Their description of antioxidant activity involved the attenuation of oxidative damage not only by scavenging radicals but also by sequestering metal ions, decomposing hydrogen peroxide and/or hydroperoxides, quenching active prooxidants and repairing damage (Niki and Noguchi, 2000). Gutteridge (1999) defined antioxidants to control the prevailing relationship between redox conditions in biological systems and recognized three levels of classification viz., i) a ‘primary’ defence that would prevent radical formation; ii) a ‘secondary’ defence would remove or inactivate the formed reactive oxygen species and iii) a ‘tertiary’ defence which would operate to remove and repair oxidatively damaged molecules (Gutteridge, 1999).

Halliwell (1995) used a screening approach to rule out direct antioxidant activity *in vivo* in which the actions of compounds should be operational over a physiologically relevant concentration range and that a compound that is poorly

effective *in vitro* will not be any better *in vivo*. Eisenbrand et al. (2002) found *in vitro* systems useful to, amongst others, provide; i) rapid and effective means of screening and ranking chemicals in and from food for a number of toxicological endpoints; ii) important tools to enhance our understanding of the hazardous effects of chemicals at both the cellular and molecular level; and iii) well defined systems for studying structure-activity relationships.

Frankel and Meyer (2000) warned that the results obtained from using any methodology to evaluate natural antioxidants must be carefully interpreted according to the system and to the analytical method used in order to determine the extent and end-point of the evaluation. Each antioxidant evaluation should be carried out under various conditions of oxidation by using different methods. They listed parameters that should be considered for protocols, including; i) the use of substrates relevant to the biological system; ii) measuring relatively low levels (below 1%) of oxidation and to include both initial products and secondary decomposition products; iii) comparing antioxidants at the same molar concentration of active components using structurally related reference compounds; iv) to use total phenol content and compositional data to compare samples of crude plant extracts; and v) to quantify the performance of the antioxidant on the basis of induction period, % inhibition or rates of hydroperoxide formation or decomposition, or IC_{50} (antioxidant concentration to achieve 50% inhibition).

Antioxidants may respond in different ways to different radical or oxidant sources since multiple reaction characteristics and mechanisms as well as

different phase locations are usually involved, emphasizing the need to use more than one assay in antioxidant evaluations (Frankel and Meyer, 2000; Prior et al., 2005). An antioxidant in one system may not necessarily be an antioxidant in all systems (Halliwell, 1990). Antioxidants may also act synergistically viz., the combination of quercetin and catechin demonstrated a synergistic effect in reducing platelet formation of H_2O_2 and inhibiting platelet function by interfering with the activation of phospholipase C pathway (Pignatelli et al., 2000). Freedman et al. (2001) showed that the antioxidant and platelet inhibitory effects cannot be attributed to a particular flavonoid isolated from purple grape juice.

The First International Congress on Antioxidant Methods held in June 2004 recognized the need for a standardized antioxidant capacity method and included the following recommendations for an 'ideal' method: (i) measuring the chemistry actually occurring in potential application(s); (ii) utilizing a biologically relevant radical source; (iii) simple; (iv) using a method with a defined endpoint and chemical mechanism; (v) instrumentation should be readily available; (vi) good within-run and between-day reproducibility; (vii) adaptability of assay of both hydrophilic and lipophilic antioxidants and use of different radical sources; (viii) adaptable to "high-throughput" analysis for routine quality control analyses (Prior et al., 2005). Performance characteristics that should be considered in the standardization of an assay include (i) analytical range, (ii) recovery, (iii) repeatability, (iv) reproducibility, and (v) recognition of interfering substances (Prior et al., 2005).

On the basis of the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories, i.e. hydrogen atom transfer (HAT) reaction based assays and single electron transfer (ET) reaction based assays. The ET-based assays involve one redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction endpoint. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. HAT-based methods generally are composed of a free radical generator, an oxidizable molecular probe and an antioxidant. HAT reactions are solvent and pH dependent and are usually completed in minutes (Prior et al., 2005) HAT-and ET-based assays are intended to measure the radical or oxidant scavenging ability instead of the preventive capacity of a sample (Huang et al., 2005) and almost always occur together in all samples, with the balance determined by antioxidant structure and pH (Van Acker et al. 1996a; Prior et al., 2005). The pH values have an important effect on the reducing capacity of antioxidants. Under acidic conditions, the reducing capacity may be suppressed due to protonation of the antioxidant compounds, whereas under basic conditions, proton dissociation of phenolic compounds would enhance a sample's reducing capacity (Huang et al., 2005).

The inhibition of lipid peroxidation by radical chain breaking and the oxygen radical absorbance capacity (ORAC) is a typical example of a HAT-based assay whereas ET-based assays include the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric ion reducing antioxidant power (FRAP), the total phenols assay by Folin-Ciocalteu reagent and the 2,2-diphenyl-1-picrylhydrazyl

(DPPH) radical scavenging capacity assay (Huang et al., 2005; Prior et al., 2005).

Conditions for assays

Webb and Ebeler (2004) noted the effect of solution conditions on the solubility of flavonoid interactions with proteins and DNA. Flavonoids in dilute aqueous solutions could be affected by pH, salinity and solvent strength that affected cell-free experiments significantly and left structure-activity studies prone to misinterpretation.

Phosphate and bicarbonate buffers are commonly used in biochemistry because they are significant physiological buffers. An important fact that influenced the results of *in vitro* assays was shown by Welch et al (2002), i.e. that, in general, the rate of Fe(II) autoxidation was increased as the pH of the solution increased and that phosphate buffers promoted the oxidation of Fe(II). Ethanol, the medium of the $ABTS^{\bullet+}$ assay, and DMSO, a common solvent of flavonoids, rapidly reacted with hydroxyl radicals (Reinke et al., 2005).

Lipid peroxidation

Lipid peroxidation is a natural biological process in which highly reactive hydroxyl radicals ($^{\bullet}OH$) are generated which initiate a free radical chain reaction (Halliwell, 1990; Kandaswami and Middleton, 1994; Moridani et al., 2003). Attack of $^{\bullet}OH$ on biological molecules can proceed by addition, hydrogen abstraction or an electron transfer reaction to form carbon-centred radicals that

can subsequently react with O_2 to give peroxyl radicals, $RO_2^{\bullet}$ (Halliwell, 1990):

$$HO^{\bullet} + R\text{-}H \rightarrow H_2O + R^{\bullet} \rightarrow RO_2^{\bullet}$$

(RH: lipid molecule)

The formation of peroxyl radicals is the major chain-propagating step in lipid peroxidation (Halliwell, 1990). It has been suggested that the scavenging of $^{\bullet}OH$ and peroxyl radicals by flavonoids can impair lipid peroxidation (Kandaswami and Middleton, 1994). Radical scavengers may be active in either the aqueous or in the hydrophobic (membrane interior) phase, but Halliwell (1990) considered those scavengers that can operate in the hydrophobic interior of biological membranes the actual chain breaking antioxidant inhibitor of lipid peroxidation. Many lipid-soluble chain-breaking antioxidants could, however, also have pro-oxidant properties that accelerate the oxidative damage to non-lipid biomolecules (Halliwell, 1990).

Transition metals that play a crucial role in the initiation of lipid peroxidation are first activated by reducing agents, i.e. ascorbate or superoxide anion (Girotti, 1985). He distinguished two broad categories of peroxidation mediated by metal ions, i.e. lipid hydroperoxide (LOOH)-independent initiation and LOOH-dependent initiation. In the former case, hydroperoxides are either absent at the outset or present in amounts that are poorly competitive with H_2O_2. In the latter case, significant amounts of LOOHs are present form the outset.

Steps in LOOH-independent initiation:

$$O_2^{-\bullet} + Fe(III) \rightarrow O_2 + Fe(II)$$

$$H_2O_2 + Fe(II) \rightarrow OH^- + {}^{\bullet}OH + Fe(III)$$

Whereas possible steps in LOOH-dependent initiation entail

$$LOOH + Fe(II) \rightarrow LO^{\bullet} + OH^{-} + Fe(III)$$

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}$$

($LO^{\bullet}$ lipid alkoxyl radical; $^{\bullet}OH$ hydroxyl radical; LH polyunsaturated fatty acid; $L^{\bullet}$ alkyl radical; LOOH lipid hydroperoxide; $LOO^{\bullet}$ lipid peroxyl radical)

Halliwell (1990) suggested in metal-ion dependant systems, an added antioxidant might act not only by scavenging peroxyl radicals but also by binding iron ions and stopping them from accelerating peroxidation. These two possibilities are illustrated in Figure 2.5 and can be easily distinguished since, if the antioxidant is acting by metal binding, it will not be consumed during the reaction whereas a chain-breaking antioxidant is consumed by reaction with peroxyl radicals in the membrane. Chain-breaking antioxidants at low concentrations often introduce a lag period into the peroxidation process, corresponding to the time taken for the antioxidant to be consumed, whereas metal-binding antioxidants will give a constant inhibition throughout the reaction.

There are problems that can be encountered in the lipid peroxidation of biological membranes assay, i.e. thiobarbituric acid can also react with tissue aldehydes and sugars, but it is still a useful tool to monitor lipid peroxidation in vitro due to its sensitivity and simplicity (Buege and Aust, 1978).

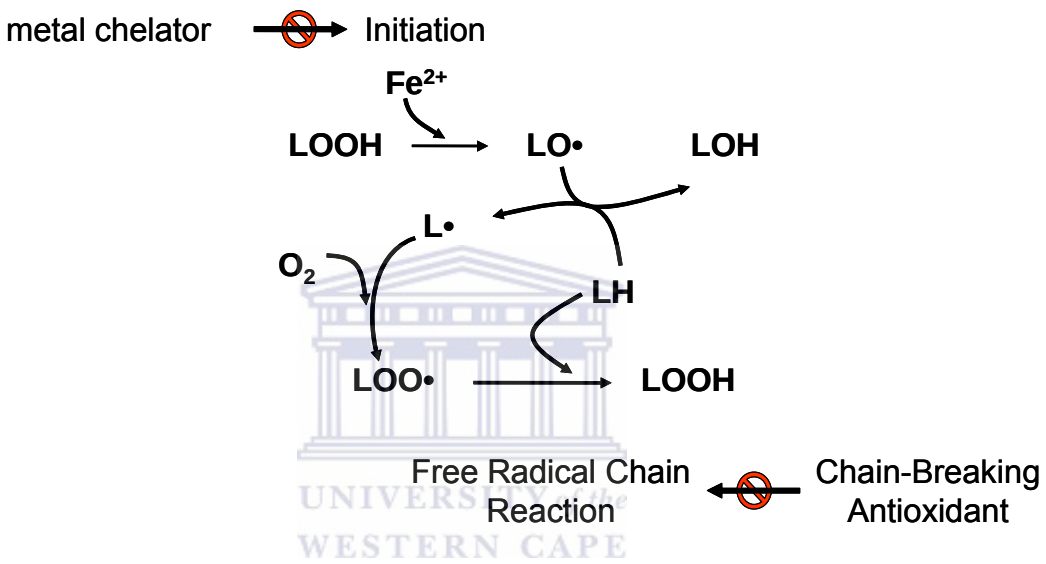


Figure 2.5. Schematic representation of the process of Fe(II)-induced lipid peroxidation and possible mechanisms of inhibition by antioxidant.

LOOH lipid hydroperoxide

LO$^{\bullet}$ lipid alkoxyl radical

$^{\bullet}$OH hydroxyl radical

LH polyunsaturated fatty acid

L$^{\bullet}$ alkyl radical

LOO$^{\bullet}$ lipid peroxyl radical

ABTS$^{\bullet+}$ assay

In the improved version of the ABTS$^{\bullet+}$ assay that was first reported by Miller et al. (1993), Re et al. (1999) involved the generation of the ABTS cation (Figure 2.6) by persulfate oxidation 12-16 hours prior to use. The solution is diluted with ethanol or buffer (pH 7.4) until the absorbance reaches 0.7 ± 0.02 at 734 nm. Ten µl of sample is mixed with 1 ml of the solution and kept at 30°C. The absorbance is read after a defined period, usually after 1 to 4 minutes has elapsed after mixing, at 734 nm,
as illustrated in Figure 2.6.

The difference of the absorbance reading is plotted against the antioxidant concentrations. The concentration of antioxidant giving the same percentage change of absorbance of the ABTS$^{\bullet+}$ as that of 1 mM Trolox was regarded as TEAC (Trolox Equivalent Antioxidant Capacity). One of the advantages of the ABTS$^{\bullet+}$ assay is that can be applied to aqueous and lipophilic systems (Re et al., 1999). It is not compounded by other factors that could contribute to the antioxidant activity in other model systems such as metal chelating and solvent partitioning (Rice-Evans and Miller, 1997). The ABTS$^{\bullet+}$ assay is a rapid assay that can be used over a wide pH range, in aqueous and organic solvents to evaluate hydrophilic and lipophilic antioxidant capacities (Prior et al., 2005). However, TEAC values for pure antioxidant compounds do not show a clear correlation with the number of electrons it can donate. Also reaction rate differences between antioxidants and oxidants are not reflected in the TEAC values because the TEAC assay is an end point assay (Huang et al., 2005).

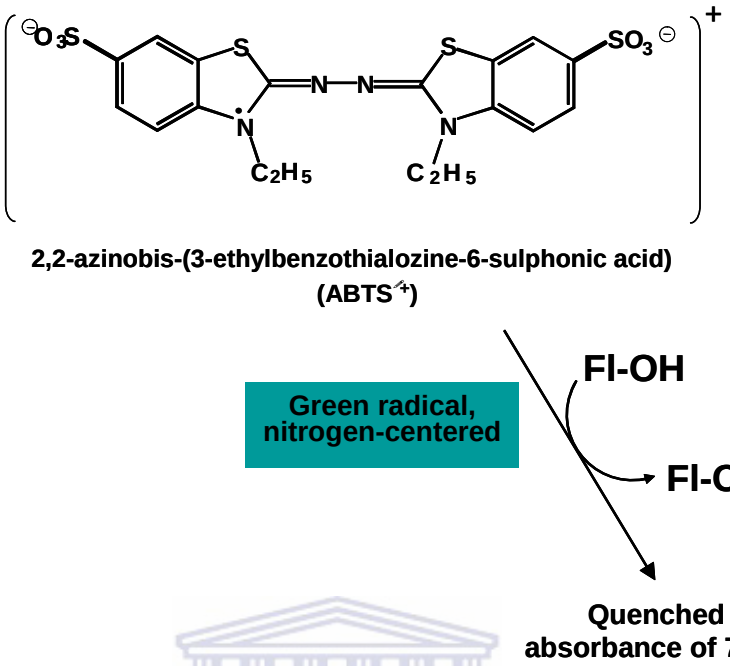


Figure 2.6. Diagrammatic representation of radical cation scavenging by a flavonoid (Fl-OH) using the $ABTS^{\bullet+}$ assay.

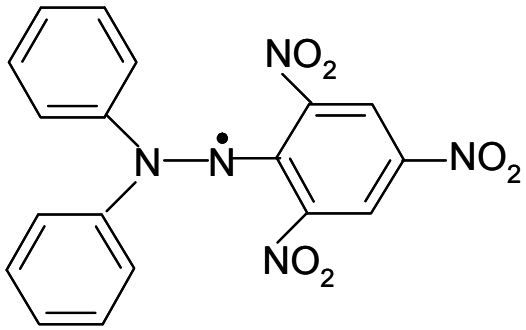


Figure 2.7. Schematic representation of the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical.

The $ABTS^{\bullet+}$ assay is, however, useful to provide a ranking order of antioxidants (Van den Berg et al., 1999).

$DPPH^{\bullet}$

The 2,2-diphenyl-1-picylhydrazyl (DPPH) radical (Figure 2.7) is a stable organic radical with a deep purple colour that was first used by Brand-Williams et al. (1995). The radical is reduced by test compounds and its consequent decrease in absorbance is monitored at 515 nm. The authors warned that any interaction of a potential antioxidant with $DPPH^{\bullet}$ is dependent on the structure of the test compound. The percentage remaining $DPPH^{\bullet}$ is calculated as an index of the antioxidant concentration and the concentration that caused a 50% decrease of the initial $DPPH^{\bullet}$ concentration is reported as an IC_{50} value. Sanchéz-Moreno et al. (1998) proposed the term antiradical efficiency (AE), that involves the product of potency ($\frac{1}{EC_{50}}$) and the reaction time ($\frac{1}{T_{EC_{50}}}$). The lower the EC_{50}, the lower the T_{EC50} and the higher the AE. A complication of the $DPPH^{\bullet}$ assay as screening method is the relative low reading wavelength. The spectra of the test compounds can easily overlap with that of the radical. The DPPH solution can discolour either via radical reaction (HAT) or reduction (SET) as well as unrelated reactions (Prior et al., 2005).

FRAP

In the FRAP assay a ferric salt, Fe(III)(2,4,6-tripyridyl-*s*-triazine)$_2$Cl$_3$, is used as oxidant under acidic conditions (pH 3.6) (Benzie and Strain, 1999). In effect it is an assay similar to the $ABTS^{\bullet+}$ assay, but with the absorbance now being monitored at 593 nm 0.5 s after the onset of the reduction and then every 15 s

thereafter until 4 min. The change in absorbance is related to the change in absorbance of a Fe(II) standard solution and is directly proportional to the concentration of the antioxidant. One FRAP unit is defined as the reduction of 1 mole of Fe(III) to Fe(II). Pulido et al. (2000) however established that the reduction caused by some polyphenols like quercetin, ascorbic acid and ferulic acid continued for several hours after the 4 min reaction time, implying that the FRAP values taken at 4 min for these compound were inaccurate. The assay does not work at physiological conditions and does not measure the contribution of liposoluble antioxidants and thiol groups (Serafini and Del Rio, 2004).

ORAC

The ORAC assay as it is used today was developed by Cao et al. (1993) and involves the reaction of the peroxyl radical with a fluorescent probe to form a fluorescent product that is quantified by fluorescence. Antioxidant capacity is measured as the area under the curve of fluorescence over time (Serafini and Del Rio, 2004), reflecting the typical radical chain breaking activity that is achieved by H atom transfer (Ou et al., 2001). It is advised that the reaction is followed for an extended period (≥ 30 min) to avoid an underestimation of antioxidant activity (Prior et al., 2005). The calculation of the protective effects of an antioxidant is from the net integrated areas under the fluorescence decay curves and accounts for lag time, initial rate and total extent of inhibition in a single value. ORAC values are usually reported as Trolox equivalents (Prior et al., 2005). However, proteins can contribute up to 86% of the total plasma TAC

value (Serafini et al., 1995) and the TAC of liposoluble antioxidants cannot be directly measured (Serafini and Del Rio, 2004).

Total phenolics/Folin Ciocalteu assay

Singleton and Rossi (1965) oxidised phenols against a gallic acid standard with a molybdotungstophosphoric heteropolyanion reagent to yield a coloured product with a λ_{max} of 765 nm. This method suffers, however, from interfering substances like sugar, aromatic amines, ascorbic acid, organic acids and Fe(II) (Prior et al., 2005). Incorrect results are also obtained due to the presence of nonphenolic organic substances such as glycine, histamine, proteins, uric acid, bensaldehyde, etc. (Peterson, 1979; Box, 1983).

Mechanisms of flavonoids for antioxidant activity

Radical Scavenging

Flavonoids are able to reduce highly oxidising free radicals such as superoxide, peroxyl and hydroxyl radicals by hydrogen atom donation as illustrated in Figure 2.8 (Husain et al., 1987; Torel et al., 1986; Robak and Gryglewski, 1988). Bors et al. (1990) described three structural determinants for effective radical scavenging by flavonoids, illustrated in Figure 2.9: (i) ortho-dihydroxy or catechol group in the B-ring which confers a high stability to the radical formed and participates in electron delocalisation; (ii) the conjugation of the B-ring to the 4-oxo group via the 2,3-double bond, which ensures the electron delocalisation from the B-ring (phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus) and (3) the 3- and 5-OH groups with the 4-oxo group, which allows electron delocalisation from both substituent

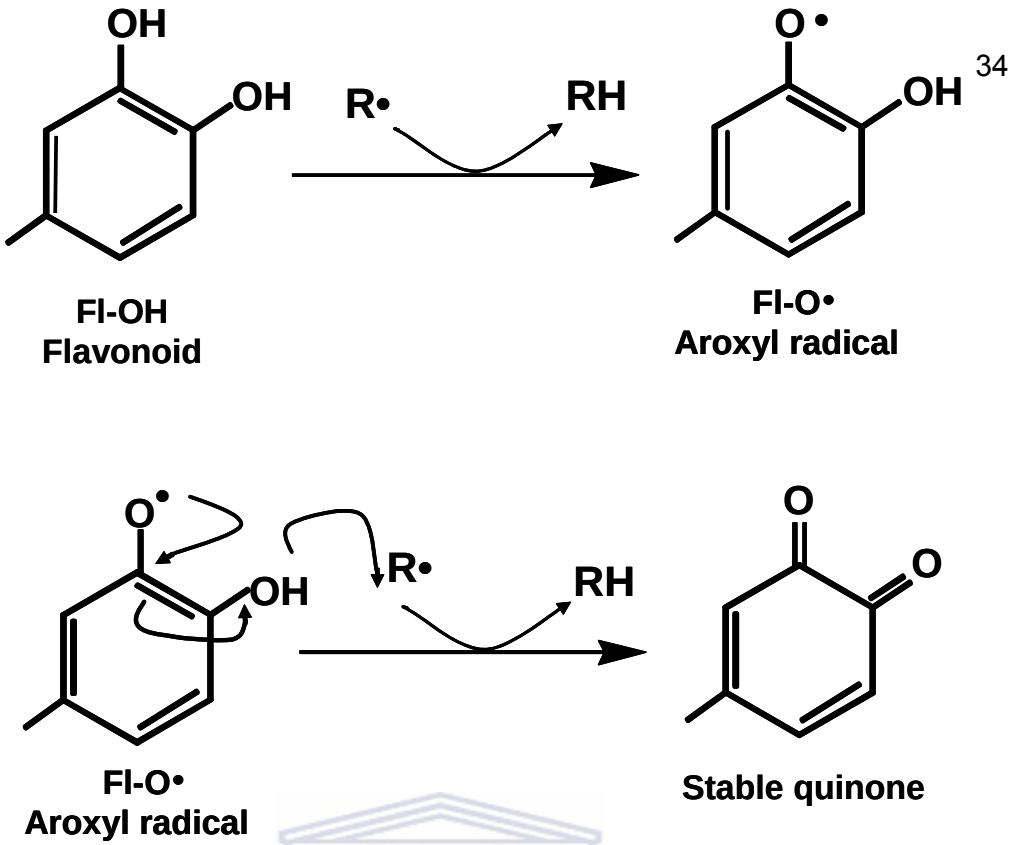


Figure 2.8. Scavenging of reactive oxygen species (R˙) by flavonoids

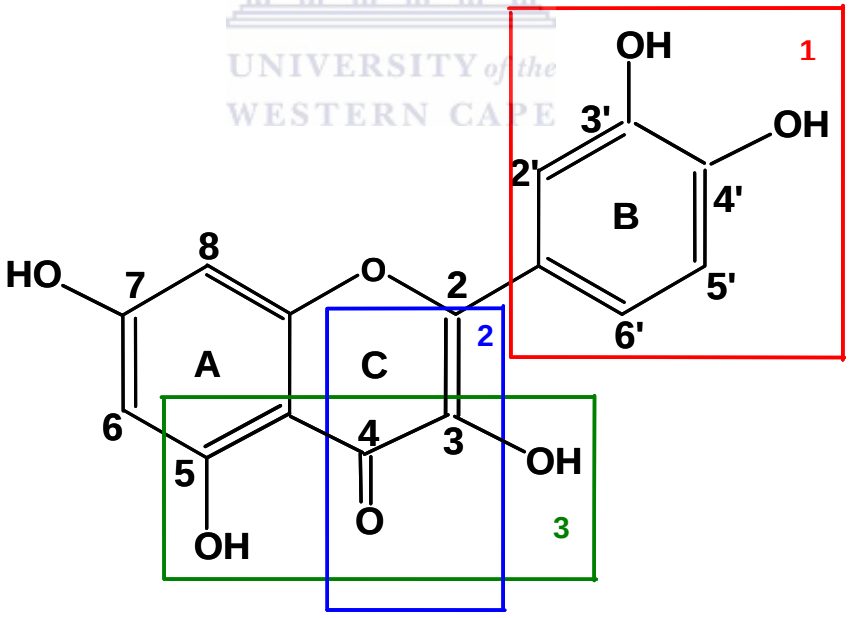


Figure 2.9. Structural requirements for effective radical scavenging by flavonoids (after Bors et al., 1990)

hydroxyl groups into the 4-keto group. The 3-hydroxyl group interacts with the B ring through a hydrogen bond between the 3-OH and the 2′ or 6′ proton which conformationally maintains the B ring in the same plane as the A and C rings thereby favouring the conjugation between the B and C ring. In flavones the B ring is slightly twisted in relation to the A and C rings.The combination of all of these structural features enables a higher degree of electron delocalisation which in turn confers a higher stability to the aroxyl radicals (Bors et al., 1990; Pietta, 2000).

The presence of a hydroxyl group at the C2´-position of a dihydrochalcone has been suggested to contribute to the radical scavenging potential of the dihydrochalcone (Nakamura et al., 2003).

Metal Chelation

Yuting et al. (1990) and Cholbi et al. (1991) identified the same three structural elements viz., 1, 2, and 3 in Fig 2.9 above which determine the effective radical scavenging properties by flavonoids as sites for metal chelation, i.e. C3-OH and C4-oxo or C5-OH and C4-oxo (sites 2 and 3 of Figure 2.9 respectively) as well as the ortho-dihydroxyl arrangement known as the catechol moiety in the B-ring (site 1) and is illustrated in Fig 2.10.

Usually, when lipid peroxidation is initiated by iron- or copper-containing systems, the radical scavenging activity of the flavonoids will prevail and the flavonoid is likely to operate by a combination of scavenging and metal complexation processes (Laughton et al., 1989; Hanasaki et al., 1994;

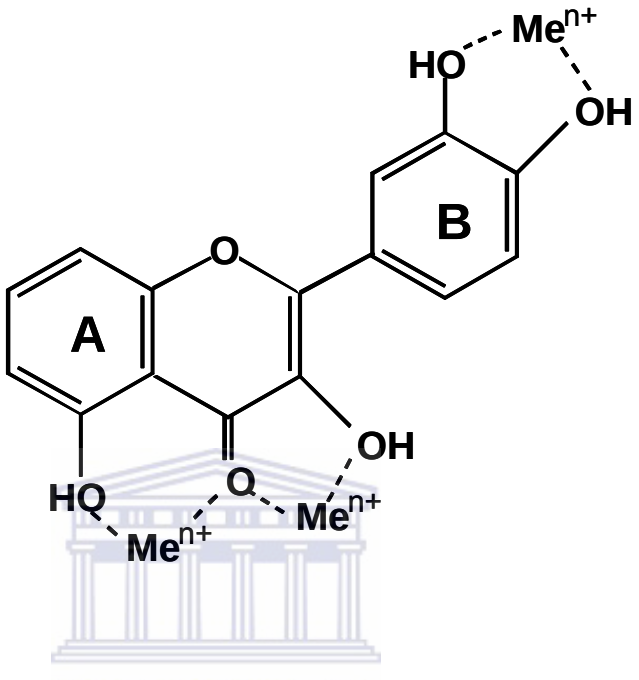
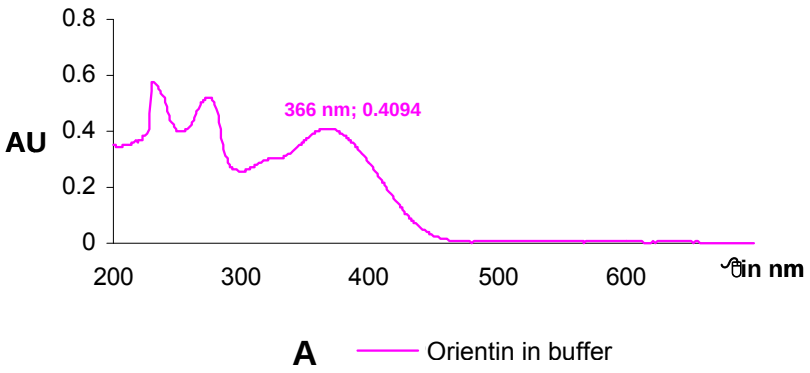


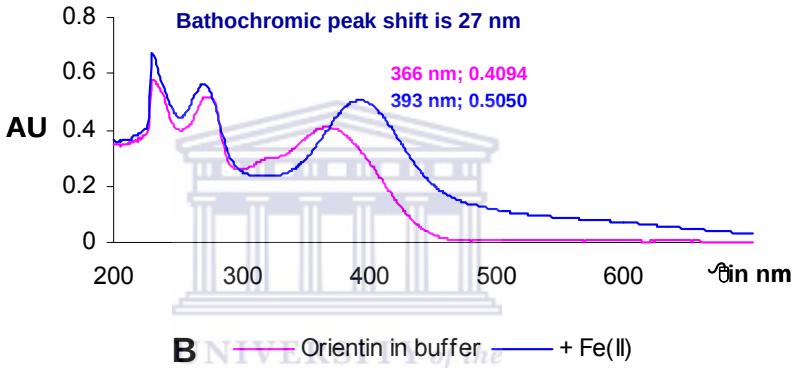
Figure 2.10. Binding sites for trace metals (after Pietta, 2000)

Afanas'ev et al., 1998). Moridani et al. (2003) actually found the flavonoid chelates to be more potent superoxide radical scavengers than their corresponding uncomplexed flavonoids in a xanthine oxidase/hypoxanthine superoxide-generating system.

The effects of Cu(II), Fe(II) and Fe(III) ion metals on the spectral characteristics are described in terms of band shifts in band I (320-420 nm) and band II (250-320 nm), which relate to B and A ring absorption, respectively (Moridani et al., 2003). These shifts are illustrated in Figure 2.11: A shows the absorption spectra recorded for orientin in buffer between 200-700 nm. B shows the band I shift after complexation between orientin and Fe(II) while C shows the recovery of orientin after ethylenediaminetetraacetic acid (EDTA), a complexing agent, has been added to abstract the chelated Fe(II) from the [orientin:Fe(II)] complex and to restore orientin.

Kaempferol with a C4′-hydroxyl on the B ring has two potential binding sites, i.e. between C4-keto and C3-OH or C5-OH, but only the one or the other will be used for chelation. Quercetin with a catechol moiety on the B ring, has three potential metal-binding sites, the same two as kaempferol as well as the catechol moiety. Hider et al. (2001) has shown that the affinity for metal chelation by the catechol moiety is greater than for two sites adjacent to the 4-keto group at pH 7. When a phenol group is conjugated with a carbohydrate moiety, it can no longer bind metals, as the dissociatable proton is lost. Rutin therefore only possesses the catechol moiety and C4-keto/C5-OH as possible metal binding sites (Hider et al., 2001).





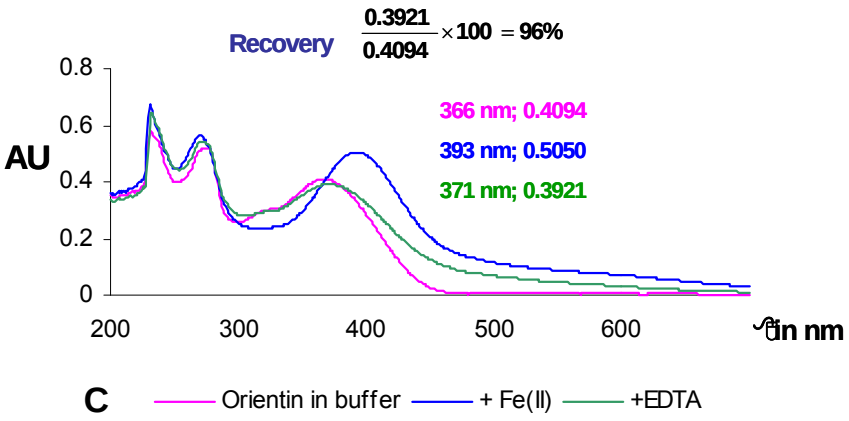


Figure 2.11. Bathochromic shifts of orientin during chelation of Fe(II)

Catechin was also able to chelate Cu(II) (Mira et al., 2002). For catechin that lacks 4-oxo, complexation must have involved the ortho-catechol group in the B ring. Morel et al. (1993) also contributed the cytoprotective effect of catechin on iron-loaded rat hepatocyte cultures to, amongst others, its iron-chelating ability.

In physiological liquids, the Fe(II) cation should be present as an aquacomplex with a variable number of coordinating water molecules (Leopoldini et al., 2006).

Binding of the transition metal ions to the biological target is a prerequisite for the $OH^{\bullet}$ radical mediated cell damage. The bound metal ion is reduced either by O_2, ascorbate or other reductants and is subsequently reoxidised by H_2O_2 yielding $OH^{\bullet}$ radicals. This cyclic redox reaction of the metal generates $OH^{\bullet}$ radicals which react with vital macromolecules with a high probability of causing 'multi-hit' damage. This 'site-specific' formation of $OH^{\bullet}$ radicals, which takes place near the target molecules, accounts both for the high damaging efficiency and for the failure of $OH^{\bullet}$ scavengers to protect against it (Samuni et al., 1983).

Vile and Winterbourn (1987) investigated iron binding to microsomes and liposomes in relation to lipid peroxidation and showed that when lipid peroxidation occurred in the absence of a chelator, a substantial proportion of the iron present was bound either to liposomes or microsomes. The way in which chelators influenced the reaction was not fully understood, but one possibility was that they altered the site where the oxidant is generated. This would occur where iron is associated with the membrane and involved a short-lived species reacting at this site before diffusing into the bulk phase. Such a

site may not be accessible to scavengers that would otherwise inhibit the reaction. Negatively charged phospholipids groups are such possible binding sites (Vile and Winterbourn, 1987).

Fe(II) was found to be present in the inner water phase and the outer bulk water phase, but not at the positively charges surface of positively charged tetradecyltrimethylammonium bromide (TTAB) micelles (Fukuzawa and Fujii, 1992). Therefore, some of the $OH^{\bullet}$ formed in the inner hydrophobic region may react with the unsaturated moiety of linoleic acid in the micelles resulting in the initiation of lipid peroxidation. However, since a radical trapping site of *N*-*t*-butyl-α-phenylnitrone (PBN) was present at the surface, this superficial PBN could not interact with $OH^{\bullet}$. At the surface, on the other hand, no $OH^{\bullet}$ was not formed because there was no Fe(II) present. When using negatively charged sodium dodecylsulfate (SDS) micelles resulting from ionic interaction with the sulphate group of SDS, positively charged Fe(II) was present at the surface and $OH^{\bullet}$ radicals were also formed on the surface. However, more radicals are present in the deeper regions of the micelles (Fukuzawa and Fujii, 1992).

Van Acker et al. (1996b) found the C3-hydroxyl group to apparently be more important for iron chelations than the C5-OH when comparing superior iron chelating ability of trihydroxyethylquercetin (C3-OH and C5-OH free) to trihydroxyethylrutin (C5-OH free). A catechol moiety seems to be more important than the C5-OH as better chelation was found by monohydroxyethylrutin (C5, C3′ and C4′ has free hydroxyl groups) than dihydroxyethylrutin where the hydroxyl on C4′ is blocked.

Partitioning

The hydrophobicity of polyphenols is intermediate between that of vitamin C (highly hydrophilic) and that of vitamin E (highly hydrophobic). Polyphenols are therefore expected to act at water-lipid interfaces (Manach et al., 2004). Glucuronidation and sulfation, metabolic processes of the liver, render polyphenols more hydrophilic and can affect their site of action and their interactions with other antioxidants (Manach et al., 2004).

Figure 2.12 shows a simplified cell membrane comprising of phospholipids that are arranged in two layers with the polar part of the molecule facing outside and the long hydrocarbon side-chains of the fatty acids on the inside to form a hydrophobic compartment.

Two possible interactions between flavonoids and lipid bilayers have been suggested by Erlejman et al. (2004), i.e. adsorption onto the membrane surface due to the interaction between the hydrophilic flavonoids with the polar head groups of lipids at the water-lipid interface and the partitioning (association) of the flavonoid according to its hydrophobic nature in the non-polar core of the membrane. A higher antioxidant capacity could be caused by the ability of flavonoids to interact with the membrane polar head groups, increasing their local concentration at the water-lipid interface of membranes (Erlejman et al., 2004).

At physiological pH, most polyphenols and their metabolites are associated with the polar heads of phospholipids at the membrane surface via the formation of

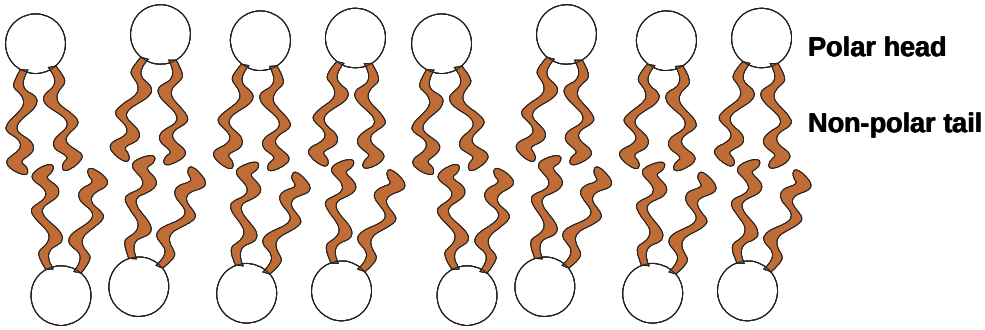


Figure 2.12. Schematic representation of a simplified cell membrane

hydrogen bonds that involve the hydroxyl groups of the flavonoids (van Acker et al., 1996b; Verstraeten et al., 2003; Manach et al., 2004) while in some lipophilic models, the membrane could be penetrated to varying degrees (Nakayama et al., 2000). A high number of hydroxyl groups on the polyphenol structure and an increase in pH can lead to deprotonation of the hydroxyl groups that would enhance interactions between the polyphenols and the membrane surface. This adsorption of polyphenols through hydrogen bonding caused an accumulation at the membrane's surface, both outside and inside the cells and probably limited the access of aqueous oxidants to the membrane surface and their initial attack on that surface. This reduced the access of deleterious molecules (i.e. oxidants) into the membranes, thus protecting the structure and function of membranes (Liao and Yin, 2000; Manach et al., 2004; Oteiza et al., 2005).

The penetration of flavonoids into the membrane is also the result of their polarity and contributes to their ability to scavenge initiating radicals: the most apolar flavone is located deeper in the membrane towards the hydrophobic core of the bilayer while increasing polarity due to an increasing number of hydroxyl groups provides the flavonoids with a higher propensity towards the aqueous phase (Silva et al., 2002; Scheidt et al., 2004). Quercetin, for instance, is known to have a deep interaction due to its planar structure (Movileanu et al., 2000). Pawlikowska-Pawlêga et al. (2003), however, showed that quercetin only influenced the polar region of the bilayer, leaving the hydrophobic core of the membrane unchanged. Its localisation close to the membrane surface protected the surface against peroxidation and because of the changes induced in the

structure, might have caused alterations in its permeability. These conclucions were confirmed by Tsuchiya et al. (2003) and Oteiza et al. (2005) who demonstrated that quercetin was the most effective antioxidant at the liposomal membrane surface, whereas EGCG was recorded to have the strongest effects in hydrophobic membrane regions because of the more favourable partition of the more non-polar compounds into the hydrophobic interior of the membrane where they exert their chain breaking antioxidant activity.

Liao and Yin (2000) also related the interaction of an agent with biomembranes, or the uptake of an agent into the membranes, strongly to its lipophilicity and expressed the relationship as a partition coefficient. They calculated partition coefficients (1 mM compound in *n*-octanol-water) for quercetin and rutin. The higher partition coefficient of quercetin to that of rutin proved that quercetin was able to interact deeper with biomembranes while having less interaction with free radicals present in the aqueous phase. These results support the suggestion that the partition coefficient of a phenolic antioxidant affects both its interaction with biomembranes and its antioxidant activity performance, particularly when water-soluble oxidants are involved (Liao and Yin, 2000). The glucoside group present in rutin made it unavailable to penetrate the model membrane, lowered its liposolubility and hampered its incorporation between the acyl chains of lipids (Liao and Yin, 2000; Saija et al., 1995). The group of Saija (1995) concluded that flavonoids anchored themselves through chemical bonds to the polar head of main phospholipids, forming reversible physico-chemical complexes depending on their liposolubility.

Flavonoids can also exert their antioxidant activity by stabilising membranes by decreasing membrane fluidity (Arora et al., 2000). The results of their study with, amongst others rutin, suggested that the flavonoids and isoflavonoids, similar to cholesterol and alpha-tocopherol, partition into the hydrophobic core of the membrane and cause a dramatic decrease in lipid fluidity in this region of the membrane. Localization of flavonoids and isoflavonoids into the membrane interiors and their resulting restrictions on fluidity of membrane components could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions. Despite the presence of polar substituents, flavonoids preferred to partition in the hydrophobic core of the membrane where they exert a membrane stabilizing effect by modifying the lipid packing order (Arora et al., 2000).

Ollila et al. (2002) opposed this above argument, i.e. that the binding of the flavonoids to or partitioning of the flavonoids into the membranes destabilises the membrane structure causing a disorientation of the membrane lipids. Their suggestion was that hydrophobic moieties of the flavonoids cause a reduction of the intermolecular hydrogen bonding capacity of water, forcing the flavonoid molecules to the more hydrophobic environment in the membranes (Ollila et al., 2002). Selected flavonoids can help maintain the integrity of the membrane by preventing the access of deleterious molecules into the hydrophobic region of the bilayer (Verstraeten et al., 2003; Manach et al., 2004).

Flavonoids can interact with both lipid and protein components of biological membranes and alter their properties. Though interactions with lipids are in

most cases limited to the polar region of the lipid bilayer; the depth of the membrane penetration by flavonoids depends on their structure. Flavonoids interact also with membrane transporter systems (Manach et al., 2004; Hendrich, 2006).

Structural activities

B ring catechol moiety

The catechol moiety in the B ring can trap free radicals, chelate redox-active metals and regenerate α-tocopherol and other antioxidants by hydrogen donation (Verstraeten et al., 2003) very efficiently as the activity of one of the hydroxyl groups is enhanced by the electron donating effect of the other one (Rezk et al., 2002).

Deng et al. (1997) studied lipid peroxidation with three alternative ways of generating the initiating radicals, i.e. γ-irradiation, Fe(II)-H_2O_2 and Fe(III)-LOOH. Their results indicated that flavonoids function as antioxidants mainly by chelating Fe(II) and scavenging peroxyl radicals rather than scavenging hydroxyl radicals. The ability of a flavonoid to complex Fe^{2+} ions by monitoring bathochromic shifts related to either A or B ring absorption was compared for rutin, quercetin, luteolin, catechin by Moridani et al. (2003).

Degree and pattern of hydroxylation

Flavonoids are antioxidants by virtue of the number and location of their phenolic hydroxyl groups attached to the ring structures and it has been determined that an increase in the number improves the reduction potential of

the flavonoids and subsequently the scavenging action is increased (Cook and Samman, 1996; Deng et al., 1997; Rice-Evans, 2001; Zhou et al., 2005). The hydroxyl group at position 3 of the AC-ring is the most reactive one and its activity is enhanced by the electron donating effect of the hydroxyl groups at positions 5 and 7 as well as the 4-keto group (Rezk et al., 2002).

The flavonols bearing *ortho*-dihydroxy groups possess significantly higher antioxidative activity than those without such adjacent functionalities and the glycosides are less active than their parent aglycones (Zhou et al., 2005). Flavonoids of different classes but with the same number of hydroxyl groups have IC_{50} values of the same magnitude in a liposome assay (Silva et al., 2002). However, the basic structure of flavonoids becomes important when the antioxidant activity of the B-ring is small (Silva et al., 2002). Chen et al. (2002) studied the relationship between the structure and hydroxyl radical scavenging activity of flavonoids in a hydroxyl radical generating chemiluminescence system with ascorbate-$CuSO_4$-H_2O_2. He concluded that (1) phenolic hydroxyls in flavonoids were the main active groups capable of scavenging $^{\bullet}OH$; (2) hydroxyl groups in rings B and A were important $^{\bullet}OH$ scavenging active groups; (3) the ortho-dihydroxyl groups in ring A and/or B could greatly enhance the $^{\bullet}OH$ scavenging activity of the rings; and (4) scavenging activity of hydroxyl groups in ring B was higher than that of hydroxyl groups in ring A (Chen et al., 2002).

Van Acker et al. (1996a) used quantum-mechanical studies to elucidate a crucial role of the C3-hydroxy group of flavonols. Their studies demonstrated that the C3-OH is capable of forming hydrogen bonds with the C2′/C6′ -

positions of the B ring facilitating the radical formation of the C4′-hydroxy group, resulting in the superior antioxidant of quercetin. The increase in radical scavenging activity due to the presence of a C3-hydroxyl group in the heterocyclic ring was also noted by Pietta et al., (2000), though additional hydroxyl or methoxyl groups at positions 3, 5, and 7 of rings A and C seem to be less important. These structural features contribute to increasing the stability of the aryloxy radical, i.e. the antioxidant capacity of the parent flavonoid (Pietta, 2000). Flavonols and flavones containing the B ring catechol moiety are highly reactive, with these flavonols being more potent than their corresponding flavones because of the presence of this C3-OH. Glycosylation of this C3-OH group viz., rutin greatly reduces the radical scavenging capacity. The presence of only one hydroxyl group would decrease the activity whereas a third group in ring B (a pyrogallol group like in epigallocatechin) would enhance the activity (Pietta, 2000).

The lone hydroxyl group in the B ring of kaempferol (C4'-OH) drastically reduced the antioxidant activity three and a half times in the $ABTS^{\bullet+}$ assay compared to quercetin which has a catechol moiety as the B ring (Rice-Evans and Miller, 1998), implying that the antioxidant activity is derived from contributions of the A and possibly the C rings.

The gross contribution to the antioxidant activity is facilitated by the delocalisation of electrons across the aromatic structure of quercetin thereby stabilizing the aryloxyl radical formed. This was shown by calculating the contribution of each group independently and adding the values for the meta-

hydroxy arrangements of the A ring in the 5,7-positions and the ortho-dihydroxy regioisomeric structural feature of the B ring as in catechin (Rice-Evans and Miller, 1998), as shown in Figure 2.13. Joubert et al. (2004) attributed the presence of only one hydroxyl group on the B ring to the poor performance of vitexin and chrysoeriol as superoxide scavenger.

Agrawal and Schneider (1983) ranked the dissociation of hydroxyl functions from 7-OH>4'-OH>5-OH while Rice-Evans and Miller (1998) found that the two controlling structural features for radical scavenging are the catechol moiety in the B ring and the 2,3 double bond in the C ring. The meta-hydroxy arrangements of the A ring in the 5,7-positions also contribute to radical scavenging.

In a study using vesicles, the relative hydrophobicity and interaction of flavonoids with artificial membranes depended on the number of hydroxyl groups. When flavones and flavanones possessing the same number of hydroxyl groups were compared it appeared that flavones were slightly more hydrophobic than flavanones. In addition the flavonols were also more hydrophobic than flavanones, but the membrane affinity of flavonols was greater due to their planar structures, making it easier for the flavonol molecule to intercalate between the phospholipids of the membrane (Van Dijk et al., 2000).

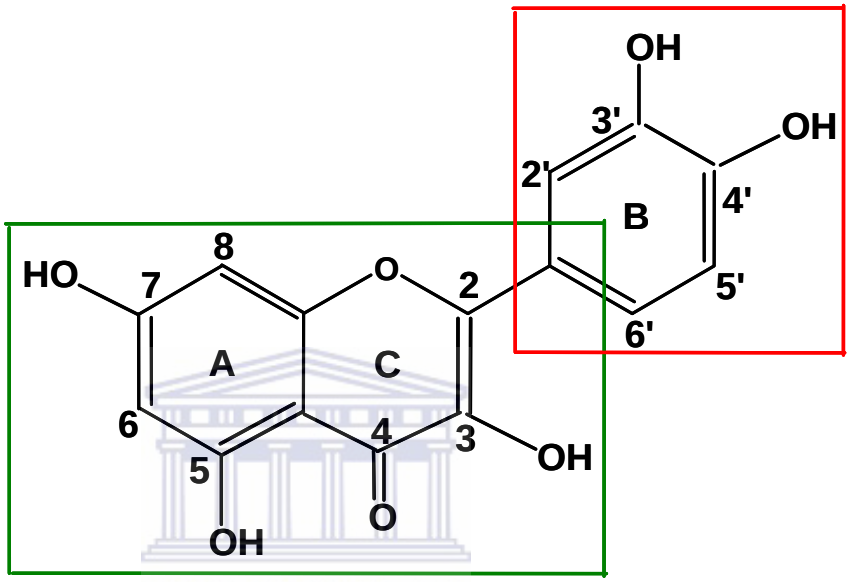


Figure 2.13. Sites for electron donation by flavonoids (after Rezk et al., 2002).

The inhibition of lipid peroxidation, as is the case with radical scavenging activity, does not dependent on the number of free hydroxyl groups present in the flavone or flavane skeleton either, but on the substitution pattern of hydroxylation (Cholbi et al., 1991). Free hydroxyl groups in the rings A (C5 or C7) and/or C (C3) participate in the inhibition of peroxidation, while the presence of hydroxyls in the B ring is not necessary, though they can increase the activity with some differences being ascribed to their positional arrangement in the molecule (Cholbi et al., 1991). However, quercetin was more active than catechin in a micellar system even though they both have the same hydroxylation pattern, but the difference in activity was attributed to increased stability of the aryloxyl radical due to the presence of the carbonyl group conjugated with ring B in quercetin (Foti et al., 1996).

Pekkarinen et al. (1999) monitored the antioxidant effect of quercetin, kaemferol, myricetin, (+)-catechin and rutin on methyl linoleate oxidation by measuring the conjugated dienes formed as well as hydroperoxide isomers. They found that the antioxidant activity of flavonoids increased with the number of phenolic hydroxyl groups. Rutin was a weak inhibitor of hydroperoxides in methyl linoleate, but quercetin was more effective than catechin because of the C ring structures (the 2,3-double bond and C4-keto group). Quercetin was better than rutin in inhibiting ketodiene formation.

Heijnen et al. (2002) observed that electron-donation weakened the O-H bond, making it easier to transfer an $H^{\bullet}$ to a lipidperoxyl radical, thus breaking the chain process of lipid peroxidation.

The structural features necessary for the inhibition of lipid peroxidation would be the presence of a C3-OH (Ratty and Das, 1988; Cholbi et al., 1991); a double bond between carbons 2 and 3 of the C ring (Mora et al., 1990); a carbonyl group on C4 (Ratty and Das, 1988); the number of hydroxyl groups (Ratty and Das, 1988; Cholbi et al., 1991) and the substitution pattern of hydroxylation (Cholbi et al., 1991) including hydroxyl groups on positions C5 and C7 of the A ring (De Whalley et al., 1990) and the catechol moiety on the B ring (Ratty and Das, 1988; Yuting et al., 1990).

Flavonols require a C2′-OH and the pyrogallol group (C-3′, C-4′, C-5′) for antiperoxidative action (Cholbi et al., 1991) while the presence of a sugar moiety reduced the antiperoxidative activity of adjacent hydroxyl groups due to steric hindrance (Ratty and Das, 1988; Cholbi et al., 1991; Mora et al., 1990). Hydrogenation of the double bond between C2 and C3 decreases antiperoxidative effects (Mora et al., 1990). The presence of methoxyl groups reduces antiperoxidative efficiency due to steric hindrance (Cholbi et al., 1991).

Flavonoids that sequester metal ions may contribute to their antiperoxidative function as well by preventing the formation of free radicals (Afanas'ev et al., 1998; Morel et al., 1993).

Methylation

Generally catechol *O*-methylation leads to a reduction in antioxidant efficacy, but the opposite has also been reported (Lemańska et al., 2004). The C4′-OH group is generally suggested to be the hydroxyl moiety primarily involved in

both deprotonation and in hydrogen donation associated with the radical scavenging action of quercetin and luteolin (Cao et al., 1997). *O*-Methylation of the C4′-OH moiety may therefore affect deprotonation as well as radical scavenging activities of the flavonoids. The results of Lemańska et al. (2004), however, showed that methylation of the C3′-OH affects both the antioxidant characteristics to almost the same extent as C4′-OH methylation. *O*-Methylation of the C4′-OH and C3′-OH position in quercetin and luteolin resulted in a decrease of their TEAC values compared to the demethylated analogues as well as compared to their aglycone form over the whole pH range tested. This effect was more pronounced for quercetin than for luteolin (Lemanska et al., 2004). Their study also showed that *O*-methylation (OH elimination) increased the pKa of the flavonoid, resulting in lower levels of deprotonation of the flavonoids at physiological pH and therefore reduced radical scavenging properties. *O*-Methylation (OH elimination) may also affect the electronic characteristics of especially the deprotonated form of the flavonoid, reducing its capacity for electron and hydrogen atom donation.

The nature and extent of methylation of the hydroxyl substitutions also affected the anti-lipoperoxidant activity of flavonoids on the mitochondrial membrane as activity was either sustained or even increased (Santos et al., 1998). Quercetin and C3′-O-methyl-quercetin, as well as 3,7,3’,4’-tetra-O-methyl-quercetin and 3,5,7,3’,4’-penta-O-methyl-quercetin were compared. Although quercetin satisfied all the structural requirements for a high antioxidant activity, 3’-O-methyl-quercetin, which carried a methyl group in the catechol moiety, was a more potent anti-lipoperoxidant. One hypothesis is that solubilization in the

mitochondrial membrane due to an increase in the lipophilic nature of molecules is implicated. In this regard, the effectiveness of protection of flavonoids against lipid peroxidation has been proposed to depend on their orientation in biomembranes, and consequently on the partition coefficients in the lipid phase (Saija et al., 1995; Rice-Evans et al., 1996; Cos et al., 2001; Heim et al., 2002). However, 3'-O-methyl catechin and 4'-O-methyl catechin, lacking metal chelating structural features were, however, less effective than catechin in protecting against low-density lipoprotein oxidation (Cren-Olivé et al., 2003).

A phenoxyl radical can also be stabilized by the electron donating ability of a methoxy group (Danilewicz, 2003), enabling the flavonoid to act as radical scavenger for a longer time, resulting in a more efficient performance as an antioxidant.

Glycosylation

In general, dietary flavonoids are attached to sugar residues which affect the mechanism of absorption by altering their physico-chemical properties and thus their ability to enter cells, rendering them consequently less biologically active, but more soluble and thus better to transport within the plant (Williamson et al., 2000; Day et al., 2003).

The antioxidant efficiency of flavonoids has been directly correlated with their degree of hydroxylation and it was found to decrease with the presence of a sugar moiety (Ratty and Das, 1988). Plumb et al. (1999), however, found that 3-*O*-glycosylation had only a small effect on inhibiting lipid peroxidation in

phosphatidylcholine vesicles. Zhou et al. (2005) also found that the glycosides are less active than their parent aglycones while Cholbi et al. (1991) concluded that in polyhydroxylated flavonoids, the blocking of active hydroxyl functions by glycosylation or methylation causes a decrease in their inhibitory behaviour due to steric effects (Cholbi et al., 1991). Glycosylation of the hydroxyl groups had a negative influence on the antiperoxidative activity of the flavonols since quercetin showed a lower IC_{50} value than isoquercitrin and rutin (Cos et al., 2001).

The physicochemical structural parameters that govern antioxidant activity are alkyl chain length, hydrophobic properties, affinity of the molecule for the lipid substrate, and structural and functionality requirements for anchoring to the phospholipid bilayer (Anselmi et al., 2004). Many phenolic aglycones are hydrophobic and can passively diffuse through biological membranes. Linkage of a phenolic OH group to a sugar moiety increases water solubility and limits passive diffusion. Quercetin glucosides are too hydrophilic to diffuse through biological membranes and thus a transport mechanism has been suggested (Williamson et al. 2000). The ability of hyperoside to protect PC12 cells against H_2O_2 and t-BuOOH induced toxicity was investigated by Liu et al. (2005). They found that hyperoside was able to permeate the cell membrane to inhibit free radical formation and the propagation of free-radical reactions by chelating transition metal ions in the cell. Yang et al. (2001) established that luteolin, rutin and hyperoside all had the same half-wave potential, but the additional sugar moiety in the case of rutin and hyperoside greatly decreased the lipophilicity resulting in a corresponding large increase in the IC_{50} value.

Glycosylation of the A ring hydroxyl groups decreased antioxidant activity (Rice-Evans and Miller, 1998). Phloridzin is the C6′-glucoside of phloretin, a dihydrochalcone with a C4-hydroxy group on the B ring. The performances of phloretin as well as phloridzin were compared in peroxynitrite scavenging and the inhibition of rat microsomal lipid peroxidation by Rezk et al. (2002). Phloretin displayed potent antioxidant properties in both the peroxynitrite scavenging and the inhibition of lipid peroxidation with IC_{50} values of 3.1 μM and 24 μM respectively. The presence of the glucose moiety increased the values to 55 and 435 μM, respectively. Orientin and isoorientin displayed strong activities towards $DPPH^{\bullet}$ and linoleic acid peroxidation, but vitexin on the other hand had a very low response (Mun'im et al., 2003). In the DPPH radical system, antioxidative substances react directly with the DPPH radical by hydrogen atom donation, similar to the mechanism of the ABTS radical scaveninging assay (Miliauskas et al., 2004). On the other hand, in the lipid peroxidation system, breaking of the chain reaction by the antioxidative compounds may have occurred.

Keto group/ C2=C3 bond

Cholbi et al. (1991) suggested that hydrogenation of the C2-C3 double bond in the C ring decreases the antiperoxidative effects of flavonoids and that the keto group at position 4 is not essential for the inhibitory activity. Bors and Saran (1987) also found that substances with the C2-C3 double bond and both 3- and 5-OH groups showed extensive resonance, which does not necessarily translate into higher radical stability.

Planarity/extended conjugation

Planarity refers to the dimensionality of the compound. Glusker and Rossi (1986) described quercetin as planar with all its hydroxyl groups in the same molecular plane. This in turn favours conjugation and delocalisation of the electrons of the whole molecule. The OH groups are arranged in such a way as to maximize the number of hydrogen bonds. Planarity is retained in going from free to complexed quercetin (Glusker and Rossi, 1986).

Cotelle (2001) as well as Silva et al. (2002) also attributed the excellent antioxidant activity of the flavonols to the conformationally planar structure that enabled the C3-hydroxyl group to interact with the B-ring through a hydrogen bond between the C3-OH and the C2' or C6' OH proton. This hydrogen bond conformationally maintained the B-ring in the same plane as the A- and C-rings favouring the conjugation between the B- and C-ring. In flavones the B-ring is twisted by c*irca* 20 degrees in relation to the plane of the A-and C-rings. Rutin with the same hydroxyl groups as luteolin had a slight decrease in activity due to loss of coplanarity of ring B with rest of molecule (Silva et al. 2002).

Anselmi et al. (2004) studied the antioxidant activity of some esters of ferulic acid with the linear fatty alcohols C7, C8 (branched and linear), C9, C11, C12, C13, C15, 16 and C18 in homogenous and heterogenous phases and found that modifications to the ester side chain (lengthening/ramification) did not influence the antioxidant potency of the molecules in a homogeneous solution (Anselmi et al., 2004). However, in the heterogeneous phase involving microsomal phospholipid bilayers, the findings were difficult to reconcile with the

differences in antioxidant potency observed. The group became aware of the possibility that the latter may be dictated by a different anchorage of the side chain with membrane phospholipids. Very likely different anchorages would affect the orientation/positioning of the radical scavenging nucleus (the phenoxy moiety) in different ways, which must be constrained outside the bilayer surface to quench the flux of free oxy radicals generated by the physiological couple Fe^{3+}/ascorbic acid.

Dihydrochalcones

Two closely related dihydrochalcones, myrigalone B (2′,6′-dihydroxy-4′-methoxy-3′,5′-dimethyl-dihydrochalcone) and angoletin (2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethyl-dihydrochalcone) were evaluated as radical scavengers in the $DPPH^{\bullet}$ assay by Mathiesen et al. (1997). Myrigalone B was an effective antioxidant while angoletin was inactive. From NMR spectra it appeared that myrigalone B showed signals consistent with a time-average conformation in which the symmetrically substituted A ring is orthogonal to the carbonyl group in the side chain. In contrast, the A ring and carbonyl group of angoletin appeared to be coplanar. By donating a phenolic hydrogen in radical scavenging, myrigalone B will lose its symmetrical structure and may change to a coplanar conformation forming a strong intramolecular hydrogen bond between the remaining phenolic hydrogen and the carbonyl group. Mathiesen et al. (1997) concluded that all the substances they investigated that had an orthogonal conformation and were able to form intramolecular hydrogen bonds by loss of a phenolic hydrogen were $DPPH^{\bullet}$ scavengers, while compounds lacking these properties were inactive.

As mentioned earlier in this chapter, the performances of phloretin as well as phloridzin were compared peroxynitrite scavenging protocols and the inhibition of rat microsomal lipid peroxidation by Rezk et al. (2002). They proposed that the stabilization of the radical that is formed after hydrogen abstraction may involve a keto-enol tautomeric transformation between the carbonyl group and the R-methylene. This explanation is confirmed by the strong activity of 2,6-hydroxyacetophenone: the free electron that is generated due to hydrogen abstraction of one of the hydroxyl groups of the AC ring can be delocalised over the three oxygen atoms present, involving a keto-enol transformation of the carbonyl group and the transfer of an α-hydrogen atom of the carbonyl group to the oxygen radical. In this way the unpaired electron is transferred to the carbonyl group. The unpaired electron can then be transferred to the other aromatic hydroxyl group by a hydrogen transfer, stabilising the radical that was formed after hydrogen abstraction The 2,4,6-hydroxyacetophenone moiety is a unique pharmacophore that could be responsible for the antioxidant activity of dihydrochalcones (Rezk et al., 2002).

The antioxidant activities of selected flavanones and their corresponding dihydrochalcones against the $DPPH^{\bullet}$ and lipid peroxidation in the erythrocyte membrane were investigated by Nakamura et al. (2003). All dihydrochalcones exhibited higher antioxidant activities than the corresponding flavanones. Their ^{1}H-NMR analysis, like that of Mathiesen et al. (1997), indicated that the active dihydrochalcone had a time-averaged conformation in which the aromatic A ring is orthogonal to the carbonyl group, while the inactive dihydrochalcone such as

2′-*O*-methyl-phloretin has a strongly hydrogen-bonded phenolic hydroxyl group, suggestive of a coplanar conformation.

A hydroxyl group at the 2′-position of the dihydrochalcone A ring, newly formed by reduction of the flavanone C ring, is an essential pharmacophore for its radical scavenging potential (Nakamura et al., 2003).

Reporting of results of *in vitro* assays

Many terms are used by different researchers to describe antioxidant capacity. Terms one can find include total antioxidant “capacity” (or efficiency, power, parameter, potential, potency, and activity). The “activity” of a chemical would be meaningless without the context of specific reaction conditions such as pressure, temperature, reaction media, co-reactants and reference points. Because the “antioxidant activity” measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay, it is inappropriate and misleading to generalize the data as indicators of “total antioxidant activity”. The other terms listed above are more independent of specific reactions and have similar chemical meanings. Oxidant-specific terms such as “peroxyl radical scavenging capacity”, “superoxide scavenging capacity”, “ferric ion reducing capacity” and the like would be more appropriate to describe the results from specific assays than the loosely defined terms “total antioxidant capacity” and the like (Huang et al., 2005).

Total Antioxidant Capacity (TAC) considers the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated

parameter rather than the simple sum of measurable antioxidants. It is the measure of the moles of a given free radical scavenged by a test solution. TAC is the result of many variables such as redox potentials of the compounds present in the matrix, cumulative and synergistic action, kind of stress, nature of oxidizing substrate and location of antioxidant; a concept rather than a technique (Serafini and Del Rio, 2004).

Total Antioxidant Activity (TAA) is calculated as the concentration of the sample being studied against the $ABTS^{\bullet+}$, expressed as ascorbic acid equivalents (mol/ℓ) (Arnao et al., 1999). Already mentioned in this chapter is Trolox Equivalent Antioxidant Capacity (TEAC: the concentration of antioxidant giving the same percentage change of absorbance of the $ABTS^{\bullet+}$ as that of 1 mM Trolox) (Re et al., 1999) and antiradical efficiency (AE: that involves the product of potency ($\frac{1}{EC_{50}}$) and the reaction time ($\frac{1}{T_{EC_{50}}}$) in the $DPPH^{\bullet}$) (Sanchéz-Moreno et al.,1998).

A useful way to report the values of inhibition dose-reponse curves, is the IC_{50} value. The IC_{50} is defined as the concentration of inhibitor (antioxidant) that provokes a response halfway between the baseline (bottom) and maximum response (top) (Addendum 3A). The IC_{50} values are often calculated from linear regression analyses (regression coefficient $\geq$ 0.9) using a minimum of three consecutive concentrations within the range of the dose-response curve. This method, however, accepts that the dose-response curve is linear, which is not the case. A typical dose-response curve is hyperbolic, becoming sigmoidal when the response is plotted against the logarithm of the dose as shown in

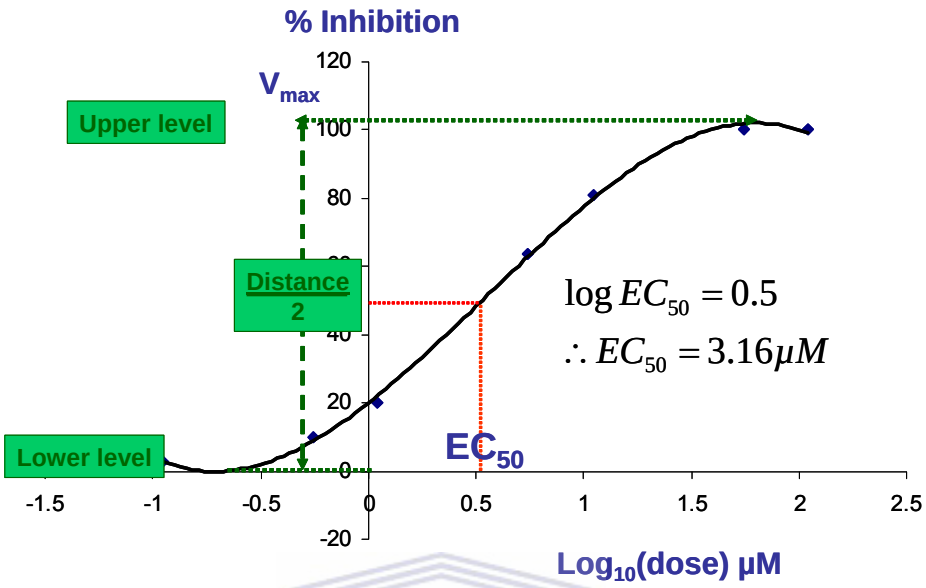


Figure 2.14. Graph illustrating the calculation of an EC_{50} value

Figure 2.14 for an EC_{50} value. EC_{50} values refer effective concentration whereas IC_{50} refer to inhibitory concentration. The same mathematical model is used to calculate both; the difference being that the slopes are in opposite directions.

Van Acker et al. (1996a) used half-peak oxidation potential to describe antioxidant activity. A low half-peak oxidation potential is defined as ready oxidizability and good scavenging.

Salmonella typhimurium mutagenicity assay

Background

The Ames *Salmonella* microsome/mutagenicity assay (*Salmonella* test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test employs several histidine dependent *Salmonella* strains each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (*his*$^+$) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner. The Ames test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs (Rosen and Stich, 1979; Mortelmans and

Zeiger, 2000). The Committee on Chemical Environmental Mutagens (1983) concluded that short-term mutagenicity tests such as the Ames *Salmonella*/microsome test has been successful in identifying carcinogens in that a substance that is found to be mutagenic in such a test a clearly a possible human carcinogen. However, cognisance should be taken of the fact that there are fundamental differences between bacterial and mammalian cells and that some reducing agents like ascorbate and selenite could induce DNA damage in mammalian cells without affecting the spontaneous mutation rate of *Salmonella* tester strains (Stich et al., 1978).

The two major kinds of point mutations are base-pair substitutions and frameshift mutations. In the former, one base pair in DNA, e.g. G:C, is replaced by another, e.g. A:T, but the number of base pairs is not altered. Unlike base-pair substitutions, frameshift mutations have gained or lost base pairs relative to the original sequence. Most commonly, frameshifts involve the gain or loss of one of two base pairs, thereby altering the reading frame of the genetic code. Frameshift mutations lead to non-functional gene products as the mutagens may stimulate the induction of mutations by reacting with DNA covalently or by non-covalent interactions. Some mutagens are electrophiles that form covalent adducts in DNA directly, others must be converted to electrophiles by mammalian metabolism (Hoffmann and Fuchs, 1997). The activation pathway of 2-acetylaminofluorene (2-AAF) and aflatoxin B_1 (AFB_1), the diagnostic mutagens for TA98 and TA100 used in this study, are shown in Figures 2.15 and 2.16 respectively.

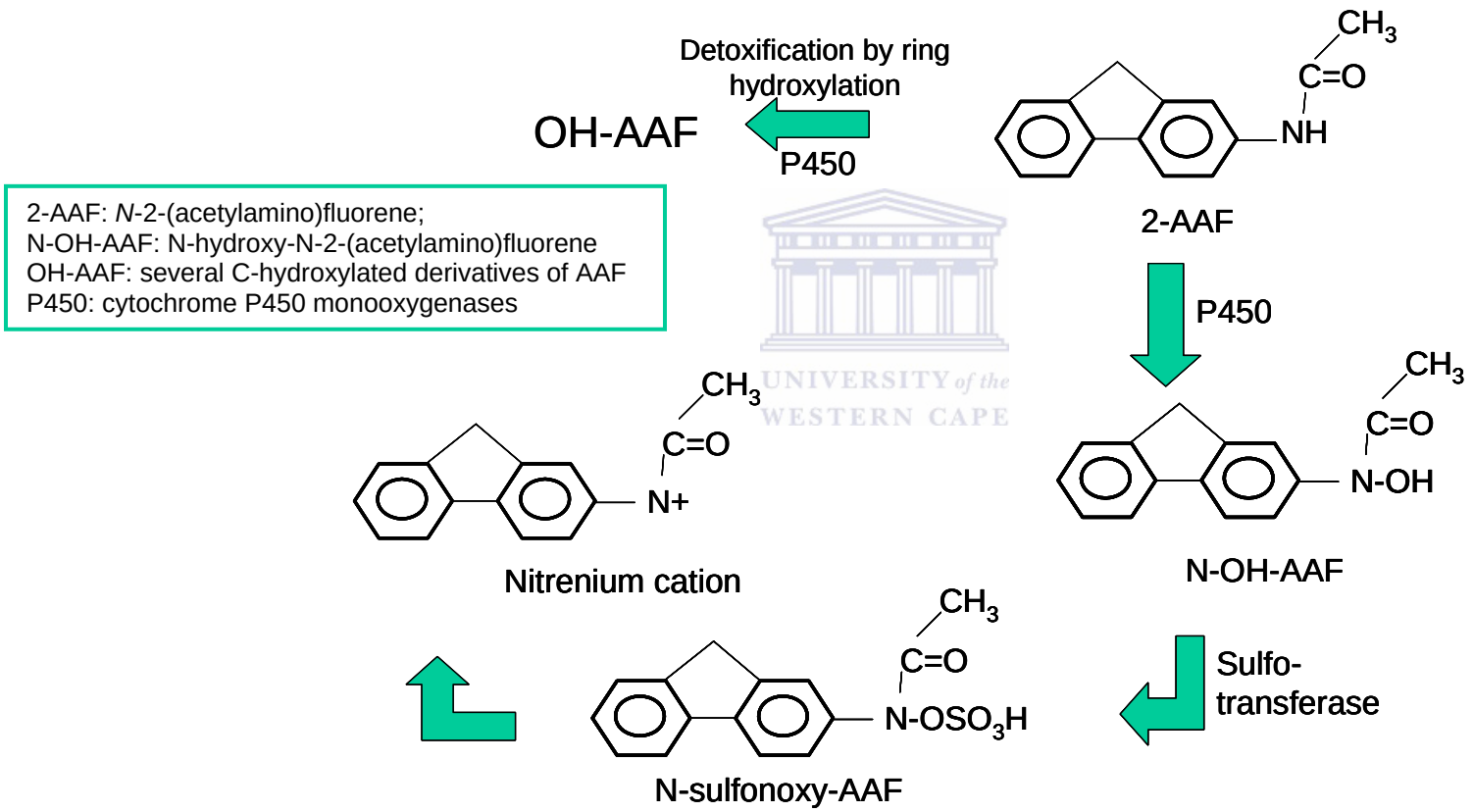


Figure 2.15. Metabolic activation of 2-acetylaminofluorene (after Heflich and Neft, 1994; Hoffmann and Fuchs, 1997).

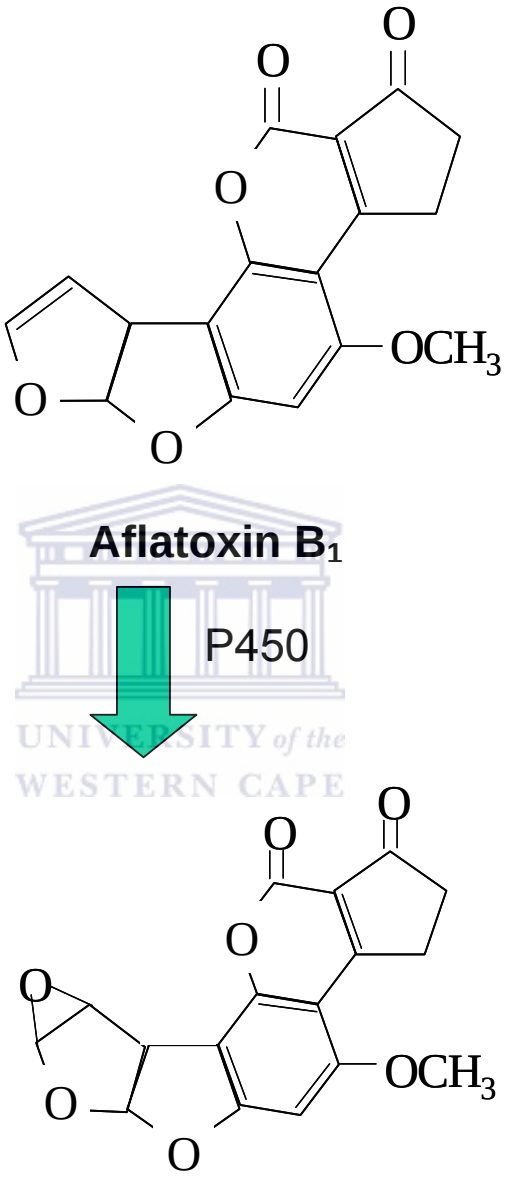


Figure 2.16. Metabolic activation of aflatoxin B_1.

Microbial methods such as the *Salmonella* test are rapid, cheap and apparently simple to carry out (Anderson and Longstaff, 1981). Another factor that favours this screening test is its reproducibility. In a study by Dunkel et al. (1985) it was concluded that the intra- and inter-laboratory reproducibility of the *Salmonella* assay with regard to the overall judgment of mutagenic or non-mutagenic was good. The solvent used for the solution of the test solvent also plays an important role as varying mutagenic responses may be obtained by using different solvents for the test compound and the responses to some compounds may be more affected than others (Anderson and McGregor, 1980).

When this assay is utilized to evaluate the antimutagenic properties of an extract or fraction, a diagnostic mutagen is utilized as positive control. Ideally, the number of reversions of the positive control should exceed that of the spontaneous reversion by 10 times (Dr WCA Gelderblom, personal communication). Some of the more popular strains of *Salmonella typhimurium* with their reversion event, targets, number of spontaneous revertants and a possible diagnostic mutagen for antimutagenicity purposes are shown in Table 2.1. Figure 2.17 shows two Petri dishes that were used in the evaluation of antimutagenicity in this study: the right hand side shows the spontaneous revertants for tester strain TA98 while the left hand side shows the positive control using TA98 with metabolically activated 2-AAF.

An artefact emanating from using the *Salmonella typhimurium* assay to evaluate antimutagenicity is that whenever the agent modulates the bacterial fission (or DNA replication), the mutation rate would appear superficially different from the

Table 2.1 Some of the most popular *Salmonella typhimurium* strains with their reversion event, targets, number of spontaneous revertants and a possible diagnostic mutagen for antimutagenicity purposes.

Strain	Reversion event	Target	Spontaneous revertants	Diagnostic mutagen
TA 97a	frameshift	–C-C-C-C-C-C- (+1 cytosine at run of C's)	90-180	2-acetamido-fluorene (2-AAF)
TA 98	frameshift	-C-G-C-G-C-G-C-G	30-50	2-acetamido-fluorene (2-AAF)
TA 100	base-pair substitution	GT ➔ AT	120-200	Aflatoxin B_1 (AFB_1)
TA102	transversion	between A and T	240-320	Cumol Hydroperoxide (CHP)

C :cytosine ; G : guanine ; T : thiamine ; A : adenosine. (after Levin et al., 1982a; Levin et al., 1982b ; Maron and Ames, 1983; Mortelmans and Zeiger, 2000)

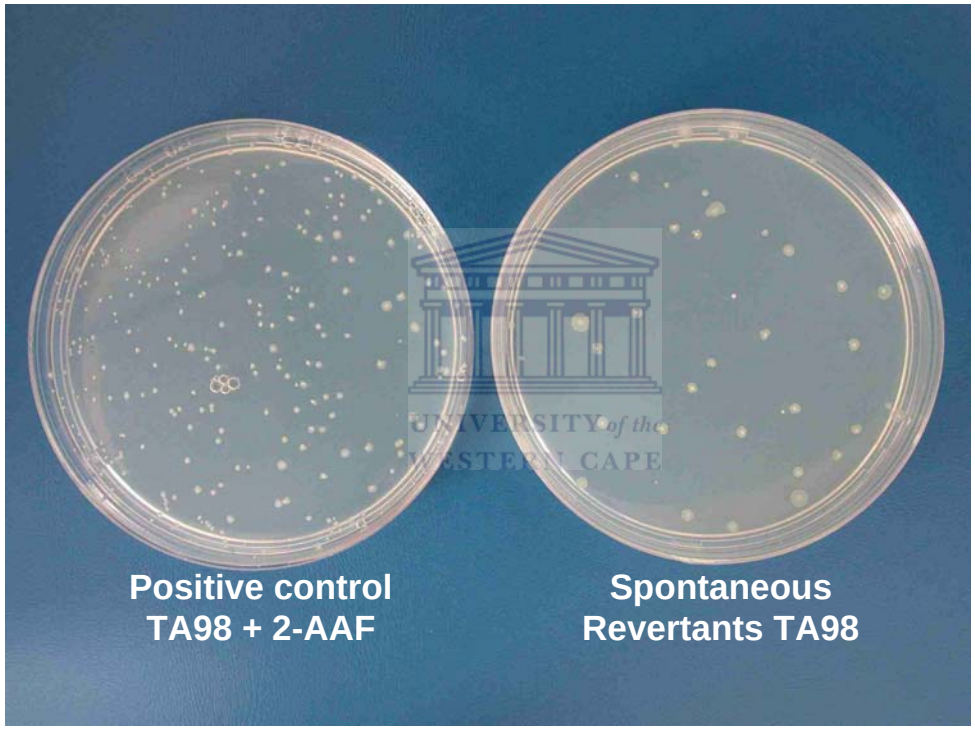


Figure 2.17. Photograph of Petri dishes with tester strain *Salmonella typhimurium* TA98. To the left is the positive control with metabolically activated 2-AAF; to the right the spontaneous revertants.

control. This means that measurements required to establish antimutagenesis must be carried out under rigorous experimental conditions because the effects induced by antimutagens are far less in the negative directions than the effects of the mutagens are in the positive direction (Liviero and von Borstel, 1996). Waters et al. (1990) added that *in vitro* antimutagenicity tests will detect only those compounds that either inhibits the metabolism of the mutagenic species directly or demonstrating an *in vitro* antimutagenic effect as *in vitro* carcinogenicity tests involve changes to the activity of one or more of the enzyme systems that are not present *in vitro*.

Rosin and Stich (1979) warned that the proximate (*N*-hydroxy-AAF) and the ultimate (*N*-acetoxy-AAF) forms of the carcinogen 2-acetylaminofluorene react differently to the antioxidants investigated and that the efficiency of the trapping agent must include testing at different stages of metabolic activation of the precarcinogen into the ultimate reactive species. There are also some limitations to the testing system, viz., antioxidants with either low water solubility like alpha-tocopherol or high toxicity like butylated hydroxyanisole are difficult to examine. They also warned against the use of DMSO as DMSO can affect the antioxidant or the carcinogen activity.

Kada and Shimoi (1987) classified antimutagens into categories of desmutagens (inactivate mutagens before they can attack DNA, thus preventing mutations as it occurs in vitro) and bioantimutagens (interfere with the expression of the mutation). Wattenberg (1983) distinguished between anticarcinogenic substances according to their mechanisms: 1. substances that

inhibit the formation of mutagens and carcinogens from their precursors; 2. substances which inhibit the arrival of carcinogens to specific cells ('blocking agents'); and 3. substances that inhibit the expression of the malignant characteristics ('suppressive agents'). The mechanisms of chemopreventive action as defined by Steele et al. (1985) were included in this list while Ramel et al. (1986) added agents that act on DNA repair and suppressing agents.

The *Salmonella* assay has been used extensively to screen plant extracts for antimutagenicity: Edenharder et al. (2001) used it in spinach (*Spinacia oleracea*), Gąsiorowski et al. (1997) on *Aronia melanocarpa* fruit, Ikken et al. (1999) on various fruit and vegetable ethanolic extracts and Nakasugi et al (2000) on Gaiyou (*Artemisoa argyi*) to name a few. Since the health properties of black and green tea (*Camellia sinensis*) have become known, extracts have also been subjected to the *Salmonella* test as shown by the work of, amongst others, Mukhtar et al. (1992), Yen and Chen (1995), Gupta et al. (2002), Santana-Rios et al. (2001) and Geetha et al. (2004).

Antimutagenicity of rooibos

Aqueous extracts of fermented and unfermented rooibos has been subjected to the *Salmonella* mutagenicity assay and showed potent antimutagenic activity against 2-AAF and AFB_1 in tester strains TA98 and TA100, respectively, with the unfermented rooibos showing more protection than fermented rooibos. Less potent inhibition was observed against peroxide-induced mutagenesis in TA102 (Marnewick et al., 2000). Standley et al. (2001) found that the antimutagenicity of rooibos was reduced during processing and fermentation. Van der Merwe et

al. (2006) showed that the antimutagenic activity of rooibos was mutagen specific and affected by fermentation and plant material, presumably due to changes and variation in phenolic composition. This group also showed that aspalathin was a moderate antimutagen in 2-AAF and aflatoxin B_1 induced mutagenesis.

Mutagenic activity of flavonoids

MacGregor and Wilson (1987) distinguished two classes of mutagenic flavones based on different structural and metabolic activation requirements. Examples of the first class are quercetin and structurally related flavonols (3-hydroxyflavones) which are active in both TA98 and TA100 strains, the activity being higher in the former. They appear to be metabolically activated to DNA-reactive intermediates, probably invoking initial oxidation of *ortho*- or *para*-hydroxyl groups in ring B to quinonoid intermediates. A free hydroxyl group at C3 seems essential. The second class consists of substituted flavones without the 3-OH group. Flavones with hydroxyl/methoxy substitutions at position 5, 7 and 8 of the A ring were most active in strain TA100 and showed only a minor or weak activity in strain TA98.

Das et al. (1994) listed molecular planarity as a requirement for mutagenicity since non-planar flavonoids exhibit weak or no mutagenicity. A C2′-OH group could distort this planarity and retard mutagenicity. They also identified two structural features determining the mutagenic activity of flavonoids, i.e. a C3-hydroxyl group or a C8-hydroxyl/methyl group. Amongst the 3-OH flavonols, the position and number of the B ring hydroxyl groups appears to be a determinant

for mutagenicity as the catechol moiety is necessary for exhibiting mutagenicity in the absence of metabolic activation. An increase in the degree of hydroxylation on the B ring reduced mutagenicity, probably due to an altered redox potential or unfavourable lipophilicity.

MacGregor and Jurd (1978) evaluated 40 structurally related compounds in TA98 and TA100 and found that the structural requirements for mutagenicity were the same for both strains, i.e a free hydroxyl on C3, a double bond at C2,C3, a C4-keto group and a structure that permits the proton of the C3-OH to tautomerise to the C3-keto. They found that the C7-OH may be responsible for metabolic action whereas the C5-OH is more important for mutagenicity. Free hydroxyl groups in the B ring were not necessary if metabolic activation was used and methylation of the C5-OH of quercetin rendered an inactive compound.

These structural requirements were confirmed in the L-arabinose forward mutation assay of *Salmonella typhimurium* of Jurado et al. (1991) who studied the mutagenicity of 13 flavonoids including quercetin, kaempferol, morin, rutin and catechin. Their data supported those previously reported for His^+ reverse mutation assays.

Duarte Silva et al. (2000) used TA98 and benzo-[a]-pyrene and considered the C2,3 double and C4-keto group to be necessary for genotoxicty of flavonoids. They also found that the presence of a free hydroxyl at C3 and the presence of a free hydroxyl on C5 of the A ring with the C7-OH were important for direct

mutagenicity. Flavonols lacking hydroxyl groups or having one group require metabolic activation to be mutagenic. Quercetin with two hydroxyl groups in the *ortho* position in the B ring does not require activation, but activation is required when the two groups are in the *meta* position.

Czeczot et al. (2000) isolated quercetin, rhamnetin, isorhamnetin, apigenin and luteolin from three medicinal herbs. The compounds were tested for mutagenicity with *Salmonella typhimurium* TA1535, TA1538, TA97, TA98, TA100 and TA102 in the presence and absence of metabolic activation. Only quercetin and rhamnetin revealed mutagenic activity with quercetin inducing point mutations in strains TA97, TA98, TA100 and TA102. Metabolic activation markedly enhanced mutagenic activity of quercetin in these strains. The group associated the following structural requirements for mutagenicity: a catechol moiety in the B ring and the presence of a free hydroxyl or methoxy group at C7 on the A ring. The presence of methoxy groups in the B ring decreased mutagenic activity of the compound.

Antimutagenic activity of flavonoids

In an experiment where 64 flavonoids were tested with respect to IQ in TA98 and towards methylated versions of IQ in TA100, there were no fundamental differences in flavonoid response between the two tester strains and no clear influence of mutagen structure on antimutagenic potency (Edenharder et al., 1993). They included that a C4-keto group of the flavane nucleus is a prerequisite for antimutagenic activity. Rings A and C of the nucleus were not essential for antimutagenicity as chalcone and three derivatives were nearly as

active as their comparable flavones. Flavanones were less potent than the corresponding flavones while dihydrochalcones and 14 structurally related saturated aromatic carbonyl compounds were inactive. Large polar substituents like sugar moieties reduced antimutagenic activity. They also considered a planar structure to be important for antimutagenicity. Another Edenharder et al. (1997) study added hydroxyl groups at C7, C3′, C4′ and C5′ to the list and considered the 3-hydroxyl group to be an interfering structural feature in the antimutagenic activity of flavonoids

The inhibitory effect of flavonoids on AFB_1 was increased by the presence of the free C5-OH and C7-OH groups, but was not affected by the saturation of the C2,C3 double bond or elimination of the C4-keto group (Choi et al., 1994)

The hydrophilic nature of flavonoids is known to be an important determinant of their antimutagenic function (Edenharder and Tang, 1997; Francis et al., 1989). The two major determinants of the hydrophilic nature of flavonoids are the hydroxylation status and the presence and position of a sugar moiety. Huang et al. (1983) has linked an increase in the number of phenolic hydroxyl groups of a flavonoid to an increased antimutagenic activity against benzo[a]-pyrene and TA100, while Edenharder et al. (1993) postulated that the increase in the number of hydroxyl groups and the subsequent increased polarity reduced the antimutagenic potential in the case of TA98 and IQ induced mutagenesis. The response was increased by the methyl etherification of hydroxyl groups. The number of hydroxyl groups could therefore either enhance or decrease the protective effect depending on the flavonoid subgroup and the type of mutagen

used. Increased hydrophilicity due to the presence of a sugar moiety could be the reason why rutin exhibited better protection against AFB_1 than quercetin (Francis et al., 1989).

Edenharder and Grünhage (2003) compared fisetin, rutin, luteolin, quercetin, isoquercitrin, hyperoxidemyricetin, myricitrin, morin, kaempferol against *tert*–butyl hydroperoxide (BHP) or cumene hydroperoxide (CHP) in TA102 and found that the number of revertants was reduced by compounds with C3′,4′-hydroxyl groups. Hydrogenation of the C2-C3 double bond with elimination of the C4-keto group caused a loss of antimutagenic activity. The same compounds were assayed with $DPPH^{\bullet}$ and the group concluded that in the *Salmonella* assay with strain TA102, the antimutagenic activities of flavonoids against the peroxide mutagens CHP and BHP are mainly caused by radical scavenging (Edenharder and Grünhage, 2003). Hatch et al. (2000), however, using the data from a quantitative structure-activity relationship data base and statistical analyses utilizing 39 diverse flavonoids, were convinced that the antioxidant or radical scavenging properties of the flavonoids are probably not involved in the inhibition of mutagenesis.

The effect of flavonoids on the metabolic pathways of mutagens and/or their reactive intermediates or the effect that flavonoids have on the Phase I (P450) activation system or Phase II detoxifying enzymes are of a biochemical nature and considered outside the field of this study. Literature on these topics were not included in this review.

Structural requirements for *in vitro* biological activities

Table 2.2 summarises the *in vitro* structural requirements of flavonoids for radical scavenging, inhibition of lipid peroxidation, mutagenic as well as antimutagenic activity. It appears that the same structures/functional groups are responsible for most of the biological activities of flavonoids, i.e. the B ring catechol moiety, C2, C3 double bond, C4 keto group, free hydroxyl groups on C3, C5 and C7 as well as a planar molecular structure. It seems therefore quite plausible that the flavonoid uses its antioxidant function, in particular the radical scavenging functions, to act as an antimutagen as Edenharder and Grünhage (2003) have suggested. It has also been shown that the mutagenic character of a flavonoid like quercetin comes into play at high dose concentrations. It appears that the trigger for a particular biological response from a flavonoid would depend on the environment of the reaction, the agonist involved as well as the concentrations of the flavonoid or agonist.

Table 2.2. Summary of general structural requirements for *in vitro* biological activity of flavonoids

Activity	B ring catechol	C2=C3	C4=O	C3-OH	C5-OH C7-OH	A ring glycosylation	C3-glycosylation	Planarity
Radical Scavenging	↑	↑	↑	↑	↑	↓	↓	↑
Antiperoxidative*	↑	↑	~	↑	↑	↓	↓	↑
Mutagenic**	↑	↑	↑	↑	↑		↓	↑
Antimutagenic** TA98/TA100	↑	↑	↑		↑		↓	↑

* against Fe(II) induced lipid peroxidation in rat liver microsomes
** as noted from responses in *Salmonella typhimurium* mutagenicity assays.
↑ denotes increased activity; ↓ denotes decreased activity; ~ no change

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CHAPTER 3

BIOGUIDED FRACTIONATION AND ISOLATION OF THE TWO MAJOR DIHYDROCHALCONES FROM UNFERMENTED ROOIBOS (*Aspalathus linearis*)

ABSTRACT

Bioactivity-guided fractionation was used to identify the most potent antioxidant and antimutagenic fractions from unfermented rooibos (*Aspalathus linearis*), as well as the bioactive principles for the most potent antioxidant fractions. The contribution of the major antioxidant, aspalathin, and its dehydroxy analogue, nothofagin, towards these biological properties of rooibos, was also investigated. Isolation procedures for aspalathin and nothofagin were also developed. The *Salmonella* mutagenicity test using tester strain TA98 and metabolically activated 2-acetoaminofluorene was used to assess the antimutagenic potential of the different extracts and fractions, while the antioxidant potency was assessed by two different *in vitro* assays, i.e. the inhibition of Fe(II) induced microsomal lipid peroxidation and the scavenging of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation.

Unfermented plant material was extracted with chloroform followed by methanol. The methanol extract was fractionated on an Amberlite XAD-2 column using an eluant comprising of methanol and water, mixed to form a stepwise gradient of decreasing polarity. Fractions were screened for the major flavonoids utilizing thin layer chromatography and pooled to yield crude phenolic fractions of different polarities.

The methanol extract exhibited higher antimutagenic and antioxidant activity than the chloroform extract. A decrease in protection against mutagenesis was observed with the less polar XAD fractions. The two XAD fractions with the lowest IC_{50} values in the antioxidant assays were further fractionated on Sephadex LH-20 and reverse phase C_{18} columns. This is the first description of the isolation of both aspalathin and nothofagin to a purity of >95%.

INTRODUCTION

The role of free radicals in the onset of cardiovascular or inflammatory diseases, cancer, aging and other disorders has become more emphasized as more information regarding the effect of these radicals on biological molecules becomes known (Clemens, 1991; Halliwell et al., 1992; Kontogiorgis et al., 2005). Free radicals are, amongst others, produced in the human body as a byproduct of oxidation (Cerutti, 1985; Halliwell et al., 1995). An antioxidant is a substance that, when present in low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell et al., 1995), thereby limiting the amount of free radicals released and allegedly delaying or preventing the onset of degenerative diseases. Recently research has increasingly focussed on natural antioxidant sources (Weisburger, 1999; Dimitrios, 2006).

Rooibos, a traditional and indigenous South African herbal drink, prepared from *Aspalathus linearis,* has been shown to be biologically active in many ways (Snyckers and Salemi, 1974; Joubert and Ferreira, 1996; Marnewick et al., 2000; Standley et al., 2001; Van der Merwe et al., 2006). This fynbos legume is

rich in aspalathin, which is unique to rooibos, and nothofagin, whose only other known source is the *Nothofagus fusca* tree (Hillis and Inoue, 1967; Joubert, 1996). Rooibos was found to be able to, amongst others, scavenge DPPH and superoxide radicals as well as inhibit lipid peroxidation (Inanami et al., 1995; Joubert et al., 2004), protect against 2-AAF) induced genotoxicity in Chinese hamster lung fibroblasts (Edenharder et al., 2002) and against 2-AAF induced mutagenesis in *Salmonella typhimurium* tester strain TA98 and TA100 (Marnewick et al., 2000; Standley et al., 2001). Aspalathin showed a strong scavenging ability against DPPH and superoxide radicals, but was a moderate antimutagen in 2-AAF and aflatoxin B_1 induced mutagenesis (Joubert et al., 2004; Van der Merwe et al., 2006).

Yen and Chen (1995) investigated the antioxidant activity and antimutagenicity of Japanese tea extracts at various stages of fermentation and found the degree of correlation depended on the antioxidant assay used, mutagen utilized and the state of fermentation of the extract. Standley et al. (2001) found that fermentation reduced not only the antioxidant activity of a rooibos extract, but also its antimutagenicity. It was hypothesized that the antioxidant and antimutagenic properties of rooibos could also be linked. Moreover, fermentation of rooibos is accompanied by a substantial decrease in the content of the two major monomeric flavonoids, aspalathin and nothofagin (Joubert, 1996). If these two dihydrochalcones were found to be important bioactive compounds of rooibos, fermentation could impair the biological activity of rooibos. It is necessary to isolate these compounds in order to assess their bioactivity in the present and future studies.

Screening tests such as the *Salmonella typhimurium* mutagenicity assay and the inhibition of lipid peroxidation in rat liver microsomes are efficient tools for identifying antimutagenic and antioxidant components. Miller and Rice-Evans (1997) described the Trolox Equivalent Antioxidant Capacity (TEAC) of a compound as a useful tool for tracking down unknown antioxidants in complex mixtures but that it should be used with care, however (Arts et al., 2004). This factor is an indicator of the ability of a compound to scavenge the 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) (Re et al., 1999).

A systematic approach in identifying bioactive principles was thus followed. An extract was selected and fractionated by column chromatography. Fractions were evaluated by thin layer chromatography against rooibos flavonoid standards and fractions with a similar profile based on Rf were grouped. The ability of each fraction to scavenge $ABTS^{+\bullet}$, and to inhibit Fe(II) induced lipid peroxidation and mutagenicity of the frameshift tester strain *Salmonella typhimurium* TA98 with the metabolically activated mutagen 2-AAF, was investigated. The phenolic acid and flavonoid content of each fraction was characterized by HPLC and its total polyphenol content determined.

MATERIALS AND METHODS

Chemicals

Chrysoeriol, orientin, isoorientin (as homoorientin), vitexin, isovitexin, hyperoside (quercetin-3-*O*-galactoside) and isoquercitrin (as quercetin-3-*O*-glucoside), were obtained from Extrasynthese (Genay, France) as HPLC grade flavonoids. Luteolin, quercetin (as quercetin dihydrate), rutin (>95%), (+)-

catechin (>98%) and epigallocatechin gallate (EGCG; >95%) were purchased from Sigma-Aldrich S.A. The mutagen in the *Salmonella typhimurium* assay, 2-AAF, was also acquired from Sigma-Aldrich S.A. Bacto agar was obtained from Difco Laboratories (Detroit, USA) and nutrient broth from Oxoid (Hampshire, UK). D-Biotin and L(-)-histidin were purchased from ICN Biomedicals Inc, (Ohio, USA) and Merck Chemicals Pty. Ltd. (Darmstadt, Germany) respectively. Solvents and chemicals such as nicotine adenine dinucleotide phosphate (NADP), glucose-6-phosphate, 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), Folin & Ciocalteu's phenol reagent, 4-methoxybenzalde-hyde and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich S.A. Chromatographic material such as BDH Amberlite XAD-2, silica gel 60 (particle size 0.063-0.200 mm/70-230 mesh) and Kieselgel TLC plates (glass plates 20 cm x 20 cm; F_{254}) were procured from Merck SA, while Bondesil reverse phase C_{18} (particle size 40 µm) and Fluka Sephadex LH-20 was obtained from Analytichem International, Harbour City, CA, USA and Sigma-Aldrich SA respectively. Closed end glass columns used for Sephadex LH-20 and C_{18} as stationary phases were from Omnifit (Separations Chromtechniques, Johannesburg, South Africa). All solvents and reagents were analytical grade, except for those used in HPLC analyses which were HPLC grade. *Salmonella typhimurium* TA98 was obtained from Prof BN Ames, Berkeley University, CA, USA. Methanol and chloroform used as column chromatographic solvents were glass-distilled prior to use unless stated otherwise. All other chemicals were used as purchased.

Preparation of Extracts

Aqueous Extract

Ground, unfermented ('green') rooibos was supplied by Dr E Joubert of the Post-Harvest & Wine Technology Division, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa. A 2% (m/v) solution was prepared in boiling water and steeped for half an hour. The solution was filtered using Whatman No. 4 filter, the extracts frozen in a methanol bath and lyophilized. The freeze-dried extract was stored in a dessicator in the dark at 4°C.

Chloroform Extract

500 g unfermented rooibos was mechanically stirred overnight in a 5 ℓ Erlenmeyer flask with 2 ℓ distilled chloroform and filtered to dryness using a double layer of Whatman No 4 filter paper. The residue was put back into the Erlenmeyer flask with 2 ℓ of fresh chloroform and stirred for 6 hours. The solution was filtered as before and the residue once again extracted with 2 ℓ chloroform overnight. After filtration, the process was repeated for another 6 hours and the suspension filtered. The dry residue was kept at 4°C. The filtrates were pooled and evaporated et vacuo. The filtrate residue was screened by TLC for the presence of aspalathin.

Methanol Extract

The dry residues of two chloroform extractions were combined and added to 4 ℓ methanol in a 5 ℓ Erlenmeyer flask. The solution was stirred mechanically for 20 hours and filtered, whereafter the residue was re-extracted with methanol for 6 hours. This procedure was repeated twice (four extractions in total). Each filtrate was evaporated to foamy dryness on a rotary evaporator and evaluated on TLC. If the phenolic profile of the four extracts corresponded, they were pooled

as the methanol extract. Care was taken that the temperature of the water bath never exceeded 40°C as aspalathin and other rooibos flavonoids are known to be sensitive to heat (Rabe et al., 1994).

Thin Layer Chromatography

Extracts and fractions of columns were analyzed by thin layer chromatography (TLC) on glass plates coated with silica gel 60 (^{254}F) for the presence of the various flavonoids. The developing solvent was $CHCl_3$:CH_3OH:H_2O:CH_3COOH: 55:36:8:1. After development, the plate was dried and viewed under ultraviolet light. Typical Rf values obtained by the rooibos flavonoids were: aspalathin 0.33; catechin 0.53; chrysoeriol 0.76; hyperoside 0.42; isoorientin 0.35; isoquercitrin 0.44; isovitexin 0.46; luteolin 0.72; nothofagin 0.41; orientin 0.39; quercetin 0.72; rutin 0.37; vitexin 0.45 (Figure 3.1). The plate was then sprayed with a 0.5% (m/v) 4-methoxybenzaldehyde solution (0.5 g 4-methoxybenzaldehyde in CH_3OH:CH_3COOH:H_2SO_4:85:10:5) (Cawood et al., 1991) and heated at 100°C for 15 minutes (Addendum 3B for copy of developed TLC plate). Column fractions were combined based on the flavonoids profile detected by TLC plates. In order to compare polarities of the flavonoids, the biologically most active flavonoid (Hu and Kitts, 2001) from *Camellia sinensis*, epigallocatechin gallate (EGCG) was evaluated under the same conditions. EGCG had an Rf value of 0.31.

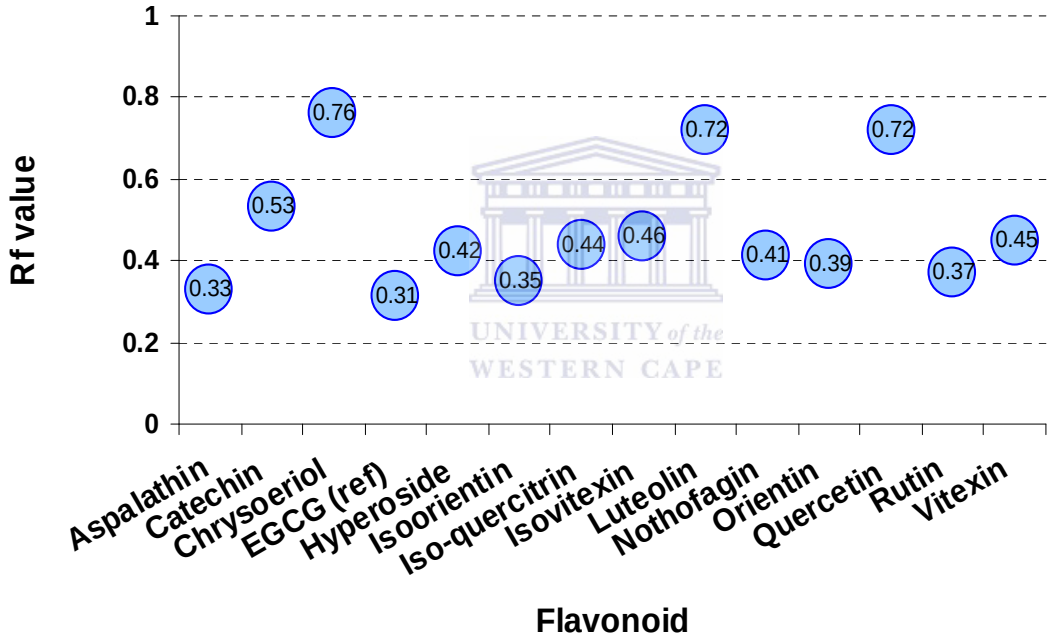


Figure 3.1. Rf values of major rooibos flavonoids, compared to that of epigallocatechin gallate (EGCG). The values were obtained on silica 60 glass TLC plate developed in mobile phase comprising of $CHCl_3$:MeOH:H_2O:HAc: 1100:720:160:20 and sprayed with a 0.5% (m/v) 4-ethoxybenzaldehyde solution

Column Chromatography

XAD-2

A crude fractionation of the methanolic extract was attained on the polymeric, non-polar adsorbant, Amberlite XAD-2. XAD-2 beads were rinsed with distilled water to remove excess chloride ions. The beads were filtered to dryness, resuspended in a 15% methanol/water solution and packed in an open glass column (65 x 770 mm). Twenty grams of the combined methanolic extracts was dissolved in 50 ml 15% methanol/water solution and applied to the column. The beaker was rinsed with 40 ml of 15% methanol/water solution which was also applied to the column. The column was run at room temperature at a flow rate of 20 ml/min and 100 ml fractions were collected. The column was eluted stepwise with a solvent gradient of decreasing polarity as shown in Table 3.1.

Table 3.1. Composition of mobile phase for XAD column

Composition	Volume (ml)
15% methanol/water	1700
25% methanol/water	2100
50% methanol/water	4000
75% methanol/water	3500
!00% methanol	1700
Rinsed with 50% methanol/chloroform	1500

Fractions were compared by TLC and fractions with corresponding bands were pooled and evaporated to dryness. Five major fractions, X1- X5, with X1 being

most polar and X5 the least polar, were collected. The percentage yield is indicated in brackets:

X1 : 0.7g (3.5%)

X2 : 8.17g (40.85%)

X3: 1.47g (7.35%)

X4: 0.79g (3.95%)

X5: 0.39g (1.95%)

The total yield amounted to 57.6%. The XAD-2 material was regenerated in a Soxhlet system by successively refluxing the beads in methanol, acetonitrile, stabilized diethyl ether and finally methanol once more. The regenerated material was stored in methanol for future use.

Isolation of aspalathin

Separation on Sephadex LH-20

Fraction X2 (Figure 3.2) was used to isolate aspalathin. A closed end column, 30 x 500 mm, was packed with Sephadex LH-20 in 50% ethanol/water. The X2 fraction (520 mg) was dissolved in 3 ml 50% ethanol/water and applied to the column. The beaker was rinsed with 1.5 ml 50% ethanol/water and the rinse was added to the stationary phase. The column was covered with a dark cloth to prevent light oxidation. A Gilson peristaltic pump was used to maintain a flow rate of 0.48 ml/min and after a pre-fraction volume of 150 ml was collected, fraction volumes of 7 ml each were collected with a Gilson fraction collector. Aliquots from the collected fractions in solution were spotted on TLC and developed as described. Fractions of which the profiles visually corresponded were pooled into four fractions:

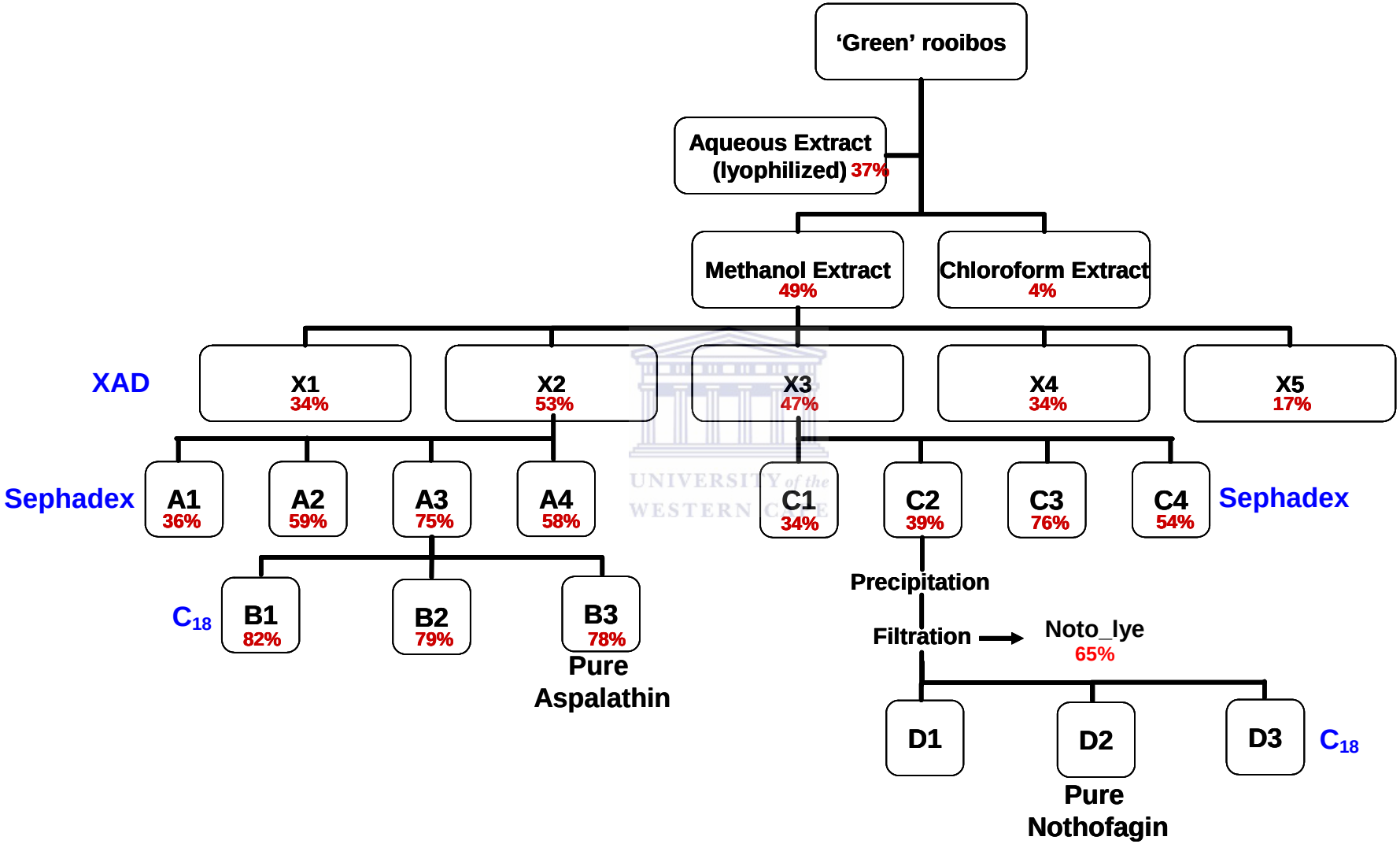


Figure 3.2. Isolation scheme for the dihydrochalcones aspalathin and nothofagin from unfermented rooibos. Numbers in red indicate total phenolics in dried extract or fraction expressed as mg Gallic Acid Equivalents/100 mg.

Pre-fractions + *f*1- *f*20 : A1 (190 mg; 36.5% yield)

*f*21- *f*36 : A2 (pre-aspalathin; 40 mg; 7.7% yield)

*f*37- *f*46 : A3 (aspalathin; 250 mg; 48.1% yield)

*f*47- *f*60 : A4 (post-aspalathin; 40 mg; 7.7% yield)

The total yield for the column was 92.3%. Sephadex was regenerated for future use by treating the packing material in a Büchner funnel successively with 300 ml each of water, methanol, iso-propanol, hexane and reversing this sequence to methanol. The stationary phase was dried in an oven at 30°C and stored in a closed container.

Separation on Reverse Phase C_{18}

Fraction A3 was used to purify aspalathin. A column, 18 x 380 mm, was packed with Reverse Phase C_{18} in 100% methanol. After the column was rinsed with 200 ml 50% methanol/acetonitrile, the column was equilibrated in 20% acetonitrile/water (200ml). A sample of fraction A3 (110 mg) was dissolved in 1 ml 20% acetonitrile/water and loaded onto the column. The beaker was rinsed with 0.5 ml 20% acetonitrile/water, which was added to the column. A flow rate of 0.8 ml/min was maintained by a Gilson peristaltic pump, running 20 ml 20% acetonitrile/water isocratically, followed by a 250 ml 20% acetonitrile/water to 250 ml 30% acetonitrile/water gradient. A pre-fraction volume of 70 ml, followed by fraction volumes of 5 ml each were collected. These were spotted and developed on TLC as described. Fractions of which the profiles visually corresponded were pooled into three fractions:

Pre-fractions + *f*1- *f*10 : B1 (2 mg; 1.8% yield)

*f*11- *f*22 : B2 (aspalathin with yellow spot; 32 mg; 29.0% yield)

*f*23- *f*28 : B3 (pure aspalathin; 30 mg; 27.3% yield)

The total yield for the column was 58.2%. C_{18} was regenerated for future use by rinsing and filtering the packing material with 300 ml each of methanol, iso-propanol and hexane and reversing the solvent order. The stationary phase was dried in an oven at 30°C and stored in a closed container.

Isolation of nothofagin

Separation on Sephadex LH-20

An aliquot of 285 mg of fraction X3 was dissolved in 3 ml of 90 % ethanol/water and applied to a 30 x 510 mm column packed with Sephadex LH-20 in the same solvent solution. The rinse of 1.5 ml was added. A flow rate of 0.44 ml/min was maintained by a Gilson peristaltic pump. After 190 ml has eluted, 8 ml fractions were collected with the aid of a Gilson fraction collector, spotted and developed. Fractions were pooled and dried as follows:

Pre-fractions + *f*1- *f*14 : C1 (83 mg; 29.1% yield)

*f*15- *f*22 : C2 (nothofagin; 55 mg; 19.3% yield)

*f23- f*30 : C3 (aspalathin; 78 mg; 27.4% yield)

*f*31- *f*50 : C4 (40 mg; 14.0 % yield)

The total yield for the column was 90%.

Precipitation

C2, in a small flask, was re-dissolved in a small volume of 20% acetonitrile/water and cooled on ice to allow precipitation. The contents were filtered with a 0.45 µm filter and washed with a small amount of cold 20% acetonitrile/water. The supernatant/filtrate was pooled and kept for future precipitation as it still contained nothofagin.

Separation on Reverse Phase C_{18}

The residue on the filter from the above procedure was dissolved in a small amount of 50% acetonitrile/water. This solvent volume was decreased on a rotary evaporator and the mixture applied to a reverse phase C_{18} column, 20 x 400 mm, equilibrated in 50% acetonitrile/water. The column was run isocratically with the same mobile phase by a Gilson peristaltic pump at a flow rate of 0.38 ml/min. Fractions of 5 ml volumes were collected and compared on TLC. Fractions were pooled according to the presence or absence of nothofagin:

*f*15- *f*22 : D1 (pre-nothofagin)

*f23- f*30 : D2 (nothofagin) (10 mg; 18% yield)

*f*31- *f*50 : D3 (post-nothofagin)

Fractions D1 and D3 yielded typically less than 2 mg each, while that of D2 depended on the mass of the residue (which was never dried prior to be loaded onto the column). The fractions containing the bulk of nothofagin were pooled as D2 and most of the solvent was removed by evaporation. The contents were freeze-dried to yield a snow white, fluffy, amorphous solid.

Evaluation of extracts and fractions

Fractions obtained from all columns were tested for antimutagenicity in the *Salmonella typhimurium* mutagenicity assay and antioxidant activity (inhibition of Fe(II) induced lipid peroxidation of rat liver microsomes and scavenging $ABTS^{+\bullet}$**)**. The total polyphenol content of the fractions was determined as well as HPLC analysis performed for a quantitative and qualitative evaluation of the phenolic profile. Active fractions were purified further.

Stock solutions

Stock solutions of extracts and fractions for use in assays were prepared in DMSO as a dilution series from 20, 10, 5 and 1 mg/ml. In the case of the antioxidant assays, a solution was diluted until the inhibition or scavenging ability of the solution was below 10%. Solutions were kept at 4°C and the same solution was used in all the assays within 5 days after preparation. Spot checks to compare the responses of fresh and week-old solutions showed no obvious differences in all the assays (data not shown). All the concentrations indicated in the results for assays as well as HPLC results are those of the original concentrations utilised.

Microsomal lipid peroxidation

Rat liver microsomes were prepared from male Fisher (F344) rats (200-300 g) as described by Shen et al. (1994). Lipid peroxidation was performed according to the method of Yen and Hsieh (1998) with slight modifications. The microsomes were diluted with a 0.2 M potassium phosphate buffer (pH 7.4) to a concentration of 1 mg protein/ml. Lipid peroxidation was induced by 5 µM ferrous sulphate (200µl) in a total volume of 1 ml, containing the different extracts/fractions dissolved in 100 µl DMSO, by incubation at 37°C for 1 hour. For each sample a sample blank was included as the compounds themselves could possibly absorb at 532 nm; any reading was subtracted from the actual sample readings. A positive control using 100 µl pure DMSO as well as reaction blank with buffer without microsomes was included. The reaction was terminated by adding 2 ml of a cold 10% trichloroacetic acid (TCA) solution

containing butylated hydroxytoluene (BHT, 0.01%) and ethylenediamine-tetraacetic acid (EDTA, 0.1%). Samples were centrifuged, 2 ml of the supernatant mixed with 0.67% thiobarbituric acid (TBA, 2 ml), heated for 20 min at 90°C and the thiobarbituric acid reactive substances (TBARS) measured at 532 nm. The percentage inhibition of TBARS formation relative to the control was calculated. The assay was conducted in threefold. IC_{50} values were calculated using GraphPad Software (Prism 5 for Windows). The fraction showing the lowest IC_{50} value was subjected to further fractionation.

ABTS Radical Cation Decolourisation Assay ($ABTS^{+\bullet}$ assay)

The 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{+\bullet}$) assay was performed as described by Re et al. (1999) with minor modifications. The bluegreen radical cation was prepared 16 h prior to the assay as the product of the reaction of ABTS (7 mM) and potassium persulphate (2.45 mM) in water and stored in the dark. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm (30°C). 1.0 ml $ABTS^{\bullet+}$ solution was added to 50 µl of the solution of the extract or fraction in DMSO, heated at 30°C for 4 minutes and the absorbance measured at 734 nm. Determinations for each concentration were done in triplicate. The percentage inhibition of absorbance displayed by the uninhibited radical cation (blank) was calculated and plotted as a function of the extract/fraction concentration. The percentage inhibition was expressed as an IC_{50} value calculated using GraphPad Software (Prism 5 for Windows).

Mutagenicity assay

The plate incorporation mutagenicity test was conducted as described by Maron and Ames (1983) and Mortelmans and Zeiger (2000) using tester strain *Salmonella typhimurium* TA 98 with 2-AAF (5 µg/plate) as mutagen. Metabolic activation was achieved by an Aroclor 1254 induced S9 homogenate (0.7 nmole cytochrome P450/mg protein), prepared from male Fischer rats (Maron and Ames, 1983), and incorporated in the S9 mixture at a level of 2 mg protein/ml. Stock solutions of the flavonoids and the mutagens were prepared, as described, in DMSO prior to conducting the mutagenic assay. The Ames assay consisted of the addition of overnight bacterial culture (100 µl) to the diagnostic mutagen (100 µl), the extract/fraction (100 µl, with a range varying from 0.001-1.2 mM per plate) and 500 µl of S9 mixture to 2 ml of top agar at 45°C. The mixture was vortexed, poured onto a minimal glucose plate and incubated at 37°C for 48 h in the dark. Positive and negative (spontaneous) controls in the presence and absence of the diagnostic mutagens respectively, as well as the presence of DMSO and S9 mixture, were included. The percentage inhibition/stimulation of the mutagen-induced response by the flavonoid was calculated using the formula $\{100-\left(\frac{(R_s-R_0)}{(R_p-R_0)}\right)x100\}$, where R_s = the average number of His$^+$ revertants induced in the presence of the flavonoid, R_0 = the average number of His$^+$ revertants in the absence of the mutagen (spontaneous revertants) and R_p = the average number of His$^+$ revertants induced by the mutagen. Five repetitions for each concentration were included.

Total Phenolic Determination

The total polyphenol content of each extract/fraction except D1, D2 and D3 was determined according to the Folin-Ciocalteau method of Singleton and Rossi (1965). Fractions from column D had too low yields. A dilution series of the extracts/fractions was prepared in DMSO as described. The Folin-Ciocalteau reagent was diluted with distilled water in the ratio 1:9. To a 1 ml Eppendorf tube, 0.5 ml of Folin-Ciocalteau solution and 0.4 ml of 7.5% Na_2CO_3 solution was added to 0.1 ml of sample solution and incubated for 2 hours at 30°C. The absorbance of the samples was read at 765 nm and absorbance readings of between 0.3 and 0.7 were used for calculations. A standard curve of gallic acid was used to determine a gallic acid equivalents value for each extract/fraction, expressed as percentage.

HPLC

The flavonoid composition of the extracts and fractions were determined by HPLC analyses according to the method by Joubert (1996), with slight modifications. A Waters LC Module1 Plus system having a quaternary pump, an autoinjector/ autosampler and a Waters 2996 photodiode array detector (Waters Corporation, Milford, MA 01757, USA), coupled with a Merck LiCrospher 100 RP-18 (5µm) column (250 x 4 mm ID) with a guard column (Merck LichroCart) was used. The column temperature was set to 30°C and the flow rate maintained at 0.4 ml/min. Column reconditioning was attained at 1 ml/min. Optimal peak separation was obtained by solvent gradient elution between mobile phases A and B, mixed in the ratio as shown in the Table 3.2.

Table 3.2. HPLC gradient elution programme to detect rooibos flavonoids (according to Joubert (1996), with minor modifications).

Time (min)	% Solvent A (Methanol)	% Solvent B (2% (v/v) formic acid in water)
0	20	80
5	20	80
25	30	70
30	31	69
40	35	65
50	40	60
60	50	50
70	60	40
80	80	20
90	60	40
110	20	80
125	20	80

Mobile phase A was pure HPLC-grade methanol (BDH HiPerSolv) while mobile phase B consisting of 2% (v/v) formic acid in deionised water (conductivity level ~ 0.1 µs/cm; filtered though activated carbon and treated by reverse osmosis) was obtained from a Modulab water purification system (Separations, Cape Town, South Africa). The solvents were additionally purified by being filtered through a 0.45 µm Millipore membrane filter (Microsep, South Africa) and

degassed in a sonicator. Aspalathin and nothofagin, as well as catechin and syringic acid peaks were quantified at 288 nm while most of the flavones under investigation and the major flavonols, as well as protocatechuic acid, p-hydroxy benzoic acid and vanillic acid were quantified at 255 nm. Finally while caffeic, p-coumaric and ferulic acids as well as vitexin and isovitexin were quantified at 320 nm. Ferulic acid co-eluted with vitexin and rutin with iso-quercitrin. Detection wavelengths were selected to closely correspond to the wavelength of maximum absorption. Peaks were integrated using the Millenium 32 software package (version 4.00) for data acquisition and system control. Stock solutions of samples of extracts and fractions were dissolved in DMSO and diluted with methanol. Sample solutions were diluted to fall within the range of the calibration curve of 5-100 µg/ml that was prepared for each standard. Samples were filtered prior to injection through a 13 mm 0.45 µm Nylon filter (Microsep). Injected volumes were adjusted from 5 to 10 µl according to sample concentration. Samples were automatically injected.

Statistical analyses

Statistical analysis was done using one-way ANOVA (Prism Version 5.00 for Windows; GraphPad Software) followed by Bonferroni's Multiple Comparison test as post test on all pairs. Data presented in columns having the same letter notation are not significantly ($P > 0.05$) different from each other.

RESULTS

Interpreting the statistical significance of IC_{50} values

In the $ABTS^{\bullet+}$ scavenging assay, at least 18 data points per extract/fraction were collected. The software was able to determine IC_{50} values with well defined lower and upper 95% confidence intervals. If the model fitted the data points, the software calculates the log IC_{50} value as well as the log standard error for the log value given. Statistical comparisons are performed on the log IC_{50} values. Significant differences shown for log IC_{50} values may be applied to the IC_{50} values (Dr Martin Kidd, Statistical Consultant, University of Stellenbosch; personal communication). The software presents this IC_{50} value with an upper and lower 95% confidence level to show the interval within which the IC_{50} can be found with 95% certainty. When computing IC_{50} values for a range of points, the customary mean value ± standard deviation cannot be used. The correct and most reader-friendly way to present these values would be to report the computed IC_{50} value showing the upper and lower levels of the 95% confidence interval with the R^2 value to show the degree of fit of the points to the model. The significance of the changes in hill slope, indicating the kinetics of an inhibition reaction, was not considered in this study.

In the case of the inhibition of lipid peroxidation, some problems regarding the data were encountered, for two reasons. The first one is that the three replicates per concentration are intra-experimental, i.e. the same solution was used to do the three repeats; three different solutions should have been used. Secondly there are for some of the samples not sufficient data points to properly define the bottom and upper plateaus, or the hill slope, of the sigmoidal dose

response curves to yield a proper fit for the model. The following were some of the identified problems:

- All X fractions gave a % inhibition higher than 100, typically 102-106% for X1 to X4, at the highest concentration (20 mg/ml) tested (data not shown). This can be attributed to absorption from the sample itself, even though readings were corrected for sample blanks. In the case of X5, however, a '% inhibition' of 133-134 was achieved at a concentration of 20 mg/ml, and 110% at 10 and 5 mg/ml, i.e. the absorbance reading read indicated a TBARS content of respectively 35% and 10% higher than the positive control. This clearly indicates a stimulation of lipid peroxidation rather than an inhibition. Although the response of X5 has not reached its top plateau, all the points with values exceeding 100 were excluded from calculations as it is % inhibition that is under investigation and that cannot exceed 100%. These omissions limited the number of data points to fit the model and the IC_{50} value for X5 was labelled as ambiguous with wide lower and upper confidence levels.
- The hill slope for A1 and C2 was very steep between two consecutive concentrations as a result of a drop in percentage inhibition from about 90 to 17 for 5 mg/ml to 1 mg/ml respectively. This resulted in the IC_{50} values for A1 and C2 also being labelled as ambiguous with wide lower and upper confidence levels.
- The IC_{50} value for the chloroform extract also had a wide interval for the lower and upper confidence levels.

These wide confidence intervals decrease the level of significant difference between extracts and fractions, and in order to show significant differences, the IC_{50} values for the inhibition of lipid peroxidation of the extracts and fractions were manually compared.

Preparation of Extracts

The chloroform [polarity index (PI) 4.1] extraction removed a major amount of lipophilic and non-polar components, extracting only a small amount of polyphenols (4%) from the unfermented rooibos (Figure 3.2). The filtrate residue was screened for the presence of aspalathin, but none was detected. After this cleanup step, extraction of polyphenols (49%) with the more polar methanol (PI 5.1) was possible. An aqueous extract (PI of water is 9), though prepared differently, yielded a polyphenolic content of only 37%. The nature of the aqueous and methanol extracts corresponded for almost all the constituents, except that methanol was able to extract the non-polar quercetin (Table 3.3). Though alike in qualitative composition, the methanol extract yielded higher concentration of the flavonoids, for example 15.26% and 2.75% (m/m) for aspalathin and nothofagin respectively in the methanol extract vs. 12.73 and 1.85% in the aqueous extracts. The aqueous and methanol extracts were equipotent ($P>0.05$) in their ability to inhibit lipid peroxidation with IC_{50} values of 0.9967 and 0.8999 mg/ml respectively. The lipophilic chloroform extract had a significantly higher IC_{50} value of 2.824 mg/ml ($P<0.05$). The methanol extract was, however, a significantly better scavenger ($P<0.05$) of the $ABTS^{+\bullet}$ than the aqueous or chloroform extracts (IC_{50} values of 0.076, 0.195 and 1.269 mg/ml

Table 3.3. Flavonoid composition of extracts/fractions of unfermented rooibos, expressed as average of at least two sample repeats ± SD*, in % (m/m).

Extract/ Fraction	Protocatechuic Acid (255)	Catechin (288)	Benzoic Acid (255)	Caffeic Acid (320)	Vanillic Acid (255)	Syringic Acid (288)	Aspalathin (288)	*p*-Coumaric Acid (320)	Orientin (255)	Isoorientin (255)	Vitexin/Ferulic acid (320)	Nothofagin (288)	Isovitexin (320)	Hyperoside (255)	Isoquercitrin/Rutin (255)	Quercetin (255)	Luteolin (255)	Chrysoeriol (255)
Aqueous	-	-	-	-	-	-	12.73±0.06	-	0.95±0.02	0.87±0.01	0.15±0.00	1.85±0.27	0.40±0.24	0.45±0.24	0.73±0.02	-	0.06±0.00	-
Methanol	-	-	-	-	-	-	15.26±0.49	-	1.11±0.07	1.12±0.06	0.17±0.01	2.75±0.11	0.36±0.02	0.67±0.24	0.75±0.06	0.09±0.00	0.10±0.00	-
X1	-	-	-	-	-	-	6.18±0.73	-	4.07±0.46	1.17±0.13	0.08±0.01	0.21±0.02	0.15±0.00	1.20±0.14	0.60±0.07	-	-	-
X2	-	-	-	-	-	-	38.12±0.05	-	2.57±0.00	2.65±0.00	0.36±0.00	3.15±0.34	0.50±0.01	1.39±0.00	1.06±0.00	-	-	-
X3	-	-	-	-	-	-	4.11±0.02	-	0.41±0.01	0.33±0.02	0.53±0.01	17.65±0.23	2.63±0.14	2.74±0.01	4.39±0.01	-	-	-
X4	-	-	-	-	-	-	0.36±0.00	-	-	-	-	0.53±0.00	0.39±0.00	0.21±0.00	0.29±0.00	-	-	-
X5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.32±0.00	-
A1	-	-	-	-	-	-	0.83±0.01	-	1.54±0.00	0.09±0.00	-	0.37±0.00	-	-	-	-	-	-
A2	-	-	-	-	-	-	10.26±0.00	-	1.18±0.00	15.05±0.01	0.92±0.04	1.90±0.00	1.67±0.00	8.55±0.01	5.09±0.01	-	-	-
A3	-	-	-	-	-	-	81.63±0.09	-	4.55±0.05	3.73±0.05	0.28±0.02	1.91±0.06	0.33±0.00	-	-	-	-	-
A4	-	-	-	-	-	-	4.46±0.00	-	1.07±0.00	0.16±0.00	-	-	-	0.73±0.04	0.82±0.01	-	-	-
B1	-	-	-	-	-	-	15.77±0.41	-	27.37±0.70	23.42±0.57	-	0.00±0.00	-	-	-	-	-	-
B2	-	-	-	-	-	-	95.67±2.01	-	2.99±0.05	0.10±0.00	-	-	-	-	-	-	-	-
B3	-	-	-	-	-	-	100.03±2.30	-	0.29±0.00	-	-	-	-	-	-	-	-	-
C1	-	-	-	-	-	-	0.15±0.11	-	0.22±0.02	0.44±0.03	0.24±0.17	3.46±2.25	1.06±0.00	1.44±0.00	0.68±0.00	-	-	-
C2	-	-	-	-	-	-	0.09±0.00	-	0.11±0.00	-	4.42±0.00	54.43±0.39	13.85±2.81	5.32±0.00	7.56±0.04	-	-	-
C3	-	-	-	-	-	-	63.16±0.04	-	4.84±0.04	6.18±0.01	-	0.63±0.10	1.68±0.00	4.21±0.04	5.48±0.04	-	-	-
C4	-	-	-	-	-	-	7.13±2.27	-	0.36±0.17	0.02±0.03	0.00±0.00	0.00±0.00	-	0.32±0.00	0.09±0.00	-	-	-
D2	-	-	-	-	-	-	0.97	-	-	-	-	99.03	-	-	-	-	-	-
Noto_lye	-	-	-	-	-	-	4.75	-	0.26	0.62	1.37	71.69	0.01	0.26	0.00	-	-	-

Flavonoids arranged in order of elution from HPLC column. Concentrations indicated are those of original solutions. Numbers in brackets indicate wavelength of detection. Coloured cell shows highest concentration of flavonoid for that extract/fraction. Cells with - : flavonoid not detected. * single injection.

respectively). The methanol extract was therefore selected for further fractionation (Figure 3.2).

XAD-2

Fractions were pooled according to similar Rf values on TLC to yield 5 fractions, X1-X5 with X1 being the most polar and X5 the least polar. X1 had a lower polyphenolic content (24%) compared to X2 (53%), X3 (47%), X4 (34%) and higher than X5 (17%) (Figure 3.2). HPLC analysis of X1 confirmed TLC results that the most abundant flavonoids were aspalathin (6.18%) and orientin (4.07%). The aspalathin content of X2 was substantially more (38.12%) while that of orientin was less (2.57%), but more isoorientin (2.65%) was present. The aspalathin, orientin and isoorientin content decreased to 4.11, 0.41 and 0.33% respectively in X3, while the lower polarity of this fraction benefited nothofagin (17.65%), isovitexin (2.63%), hyperoside (2.74%) and the isoquercitrin/rutin combination (4.39%). The combined X4 fraction yielded only small quantities (<1%) of flavonoids while X5 was the only XAD fraction that contained luteolin (1.32%). X5 contained had no other known peak.

All the XAD fractions displayed potent antimutagenic activity against 2-AAF induced mutagenesis in TA98 at a concentration of 20 mg/ml with no difference between fractions X1, X2, X3 and X4 ($P>0.05$) (Table 3.4). Fraction X1 displayed the highest activity at the lower concentrations evaluated ($P<0.05$). Fraction X3 had the lowest IC_{50} value (0.1098 mg/ml) (Table 3.5; Figure 3.3) and fraction X1 the highest (0.5851 mg/ml) in the lipid peroxidation assay ($P<0.05$). The most efficient inhibitor ($P<0.05$) of $ABTS^{\bullet+}$ was fraction X2 (35

Table 3.4. % Inhibition (-) or stimulation (+) of the mutagenic response of 2-AAF by various extracts and fractions against tester strain *Salmonella typhimurium* TA98 in the presence of metabolic activation.

			Concentration of tester solution in mg/ml			
		Extract/Fraction	**20**	**10**	**5**	**1**
		Aqueous	(-)98±1**a**[1]	(-)73±4**a**[2]	(-)32±6**a**[3]	(-)19±3**a**[3]
		Methanol	(-)99±1**a**[1]	(-)98±1**b**[1]	(-)86±6**b**[2]	(-)7±4**b**[3]
		Chloroform	(-)69±5**b**[1]	(-)27±5**c**[2]	(+)10±8**c**[3]	(+)61±6**c**[4]
	XAD	**X1**	(-)101±0**a**[1]	NT	(-)97±2**b**[1]	(-)27±6**ad**[2]
		X2	(-)99±2**a**[1]	NT	(-)62±8**f**[2]	(-)16±2**a**[3]
		X3	(-)98±2**a**[1]	NT	(-)60±4**f**[2]	(-)13±4**ab**[3]
		X4	(-)96±2**a**[1]	NT	(-)2±12**chjk**[2]	(+)40±30**cf**[2]
		X5	(-)89±2**c**[1]	NT	(-)12±10**hijk**[2]	(+)126±11**e**[3]
Aspalathin	**Sephadex**	**A1**	NT	(-)49±5**d**[1]	(-)48±4**d**[1]	(-)24±3**ad**[2]
		A2	NT	(-)54±3**d**[1]	(-)50±2**d**[1]	(-)28±3**d**[2]
		A3	NT	(-)29±5**ce**[1]	(-)25±3**ae**[1]	(-)26±2**d**[1]
		A4	NT	(-)80±2**a**[1]	(-)70±3**f**[2]	(-)23±2**ad**[3]
	C_{18}	**B1**	NT	(-)47±3**d**[1]	(-)35±4**a**[2]	(-)17±2**a**[3]
		B2	NT	(-)22±3**ef**[1]	(-)23±3**eg**[1]	(-)18±3**a**[1]
		B3	NT	(-)16±4**cf**[1]	(-)15±4**gh**[2]	(-)7±2**b**[2]
Nothofagin	**Sephadex**	**C1**	NT	(-)32±1**c**[1]	(-)18±2**egi**[2]	(-)3±1**b**[3]
		C2	NT	(-)17±5**cf**[1]	(-)18±4**egj**[1]	(-)17±4**a**[1]
		C3	NT	(+)2±4**g**[1]	(-)16±3**gk**[1]	(-)16±2**a**[2]
		C4	NT	(-)99±1**b**[1]	(-)88±2**b**[2]	(-)19±2**ad**[3]
		Noto_lye	NT	(+)89±2**h**[1]	(+)63±3**l**[2]	(+)15±5**f**[3]

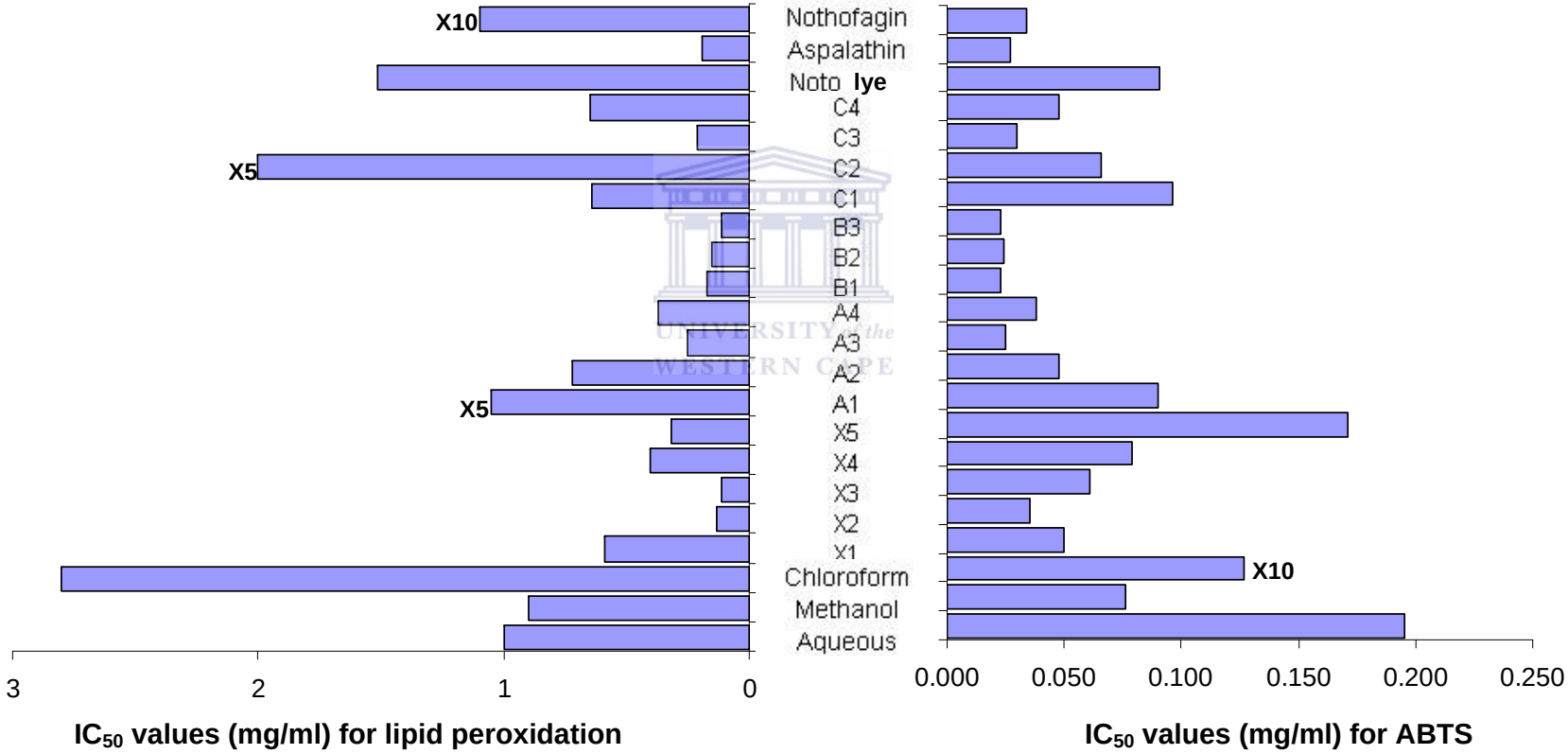
Values are the means±SD of 5 plates per treatment. Values in columns followed by the same letter do not differ significantly (P≥0.05); if letters differ, then P<0.05. Values in rows followed by the same superscript number do not differ significantly (P≥0.05); if numbers differ, then P<0.05. Average spontaneous revertants (n=40) are 35±6; 2-AAF positive control 397±33; spontaneous revertants subtracted from response to calculate % inhibition. Concentration of 2-AAF: 5µg/plate. Concentration of compound indicated as mg/ml of original solution tested. NT=not tested.

Table 3.5. Results of the inhibitory performance of the various extracts and fractions of unfermented rooibos in the lipid peroxidation (TBARS) and ABTS radical cation scavenging antioxidant assays, expressed as IC_{50} values, in mg/ml.

		Extract/ Fraction	Lipid peroxidation			$ABTS^{+\bullet}$	
			IC_{50} value (mg/ml)	R^2	Number of points analyzed	IC_{50} value (mg/ml)	R^2
		Aqueous	0.9967 (0.8575; 1.159) **a**	0.9894	18	0.156 (0.123; 0.197) **a**	0.9944
		Methanol	0.8999 (0.7699; 1.052) **a**	0.9810	18	0.089 (0.074; 0.106) **b**	0.9900
		Chloroform	2.824 (1.992;4.005) **b**	0.9869	18	1.221 (0.951; 1.568) **c**	0.9950
	XAD	X1	0.5851 (0.5684; 0.6024) **c**	0.9992	12	0.060 (0.048; 0.077) **d**	0.9971
		X2	0.1308 (0.1182; 0.1448) **d**	0.9980	15	0.051 (0.048; 0.055) **d**	0.9989
		X3	0.1098 (0.0542; 0.2226) **d**	0.9997	15	0.077 (0.074; 0.081) **e**	0.9996
		X4	0.4033 (0.3363; 0.4836) **e**	0.9984	12	0.103 (0.092; 0.118) **f**	0.9969
		X5	0.3198 (wide; wide) **f**	0.9966	9	0.171 (0.141; 0.207) **g**	0.9992
Aspalathin	**Sephadex**	A1	5.251 (wide; wide) **g**	0.9989	18	0.092 (0.086; 0.097) **h**	0.9926
		A2	0.7194 (0.6850; 0.7554) **h**	0.9985	15	0.055 (0.052; 0.058) **i**	0.9945
		A3	0.2457 (0.2089; 0.2891) **i**	0.9975	15	0.036 (0.033; 0.039) **j**	0.9783
		A4	0.3709 (0.3166; 0.4246) **j**	0.9955	15	0.044 (0.042; 0.047) **k**	0.9930
	C_{18}	B1	0.1713(0.1268; 0.1536) **k**	0.9983	15	0.029 (0.027; 0.031) **l**	0.9873
		B2	0.1481 (0.1286; 0.1706) **k**	0.9969	15	0.032 (0.028; 0.036) **l**	0.9841
		B3	0.1137 (0.0823; 0.1572) **k**	0.9929	15	0.029 (0.025; 0.032) **l**	0.9846
Nothofagin	**Sephadex**	C1	0.6479 (0.6152; 0.6824) **l**	0.9963	18	0.102 (0.096; 0.109) **m**	0.9917
		C2	>10(wide; wide) **m**	0.9980	12	0.074 (0.070; 0.078) **n**	0.9926
		C3	0.2113 (0.1851; 0.2412) **n**	0.9980	15	0.041 (0.038; 0.037) **p**	0.9875
		C4	0.6451 (0.6054; 0.6874) **l**	0.9959	15	0.058 (0.056; 0.062) **q**	0.9902
		Noto_lye	1.373 (1.131; 1.668) **o**	0.9939	18	0.104 (0.100; 0.109) **r**	0.9914

Values are IC_{50} values with lower and upper 95% confidence limit of 3 replicates per treatment. Values in columns followed by the same letter do not differ significantly (P>0.05). Fraction X2 was used for Sephadex column in aspalathin isolation; fraction X3 for nothofagin. Concentration indicated that of original solution used in assays.

Figure 3.3. Graph showing IC_{50} values in mg/ml of the different extracts and fractions for (A) inhibition of lipid peroxidation and (B) the ABTS•+ scavenging activity. Values are calculated from three determinations.



µg/ml) and the least efficient scavenger fraction X5 (IC_{50} of 0.171 mg/ml). Fractions X2 and X3 were selected for further fractionation.

Isolation of aspalathin

The Sephadex LH-20 column, used for the further separation of X2, was effective in separating a major contaminant, isoorientin, from aspalathin. The possible chemical transformation of aspalathin into orientin and isoorientin, induced by light and heat, hampered the isolation process of aspalathin as the Rf values of orientin and isoorientin, 0.39 and 0.35 respectively, are very close to that of aspalathin (0.33). Fraction A2 (59% total polyphenols) contained 10.26, 1.18 and 15.05 % (m/m) of aspalathin, orientin and isoorientin, respectively, whereas these respective compounds constituted 81.63, 4.55 and 3.73% of fraction A3 (75% total polyphenols). Fraction A3 also had the lowest IC_{50} values in both the lipid peroxidation and $ABTS^{\bullet+}$ scavenging assays ($P<0.05$) and was therefore applied to a reverse phase C18 column for purification. Three fractions were collected of which B2 (79% total polyphenols) contained 95.67% (m/m) aspalathin with 2.99% (m/m) orientin still visible on TLC. Fraction B3, however, yielded a cream-coloured amorphous dried powder that consisted of ~99% (m/m) aspalathin with only 0.29% orientin and no isoorientin detected. The total polyphenol content for B3 was calculated as 78%. This fraction also displayed the lowest IC_{50} values for this column in the lipid peroxidation ($P<0.05$) and $ABTS^{\bullet+}$ scavenging ($P>0.05$) assays. Efforts to crystallise aspalathin were unsuccessful. The purity of the isolated aspalathin was established to be >95% based on TLC and HPLC (Addendum 3C), verified

by independent LC-MS (Addendum 3D) and NMR (Addendum 3E) analyses. The biological activity of aspalathin is reported in Chapters 4 and 5.

Isolation of nothofagin

Fraction X3 (47% total polyphenols) contained 17.65% (m/m) nothofagin and displayed the lowest IC_{50} value for lipid peroxidation, i.e. 0.1098 mg/ml, of all the extracts or fractions ($P<0.05$), while it was almost half as efficient an inhibitor of $ABTS^{\bullet+}$ than X2 (0.061 vs 0.035 mg/ml) ($P<0.05$). X3 was fractionated into four fractions that contained 34% (C1), 39% (C2), 76% (C3) and 54% (C4) total polyphenols, respectively (Fig. 3.2). The high polyphenolic content of C3 could be attributed, amongst others, to 63.16% aspalathin, 5.48% rutin/isoquercitrin and 6.48% isoorientin and resulted in C3 having the highest antioxidant potency of the column C fractions for both assays ($P<0.05$). Fraction C2 consisted of, amongst others, 54.43% nothofagin, 13.85% isovitexin, 7.56% isoquercitrin/rutin, 5.32% hyperoside and 4.42% vitexin/ferulic acid, representing only 39% total polyphenols. Rf values reflected their similar polarities and varied from 0.46 for isovitexin to 0.41 for nothofagin. Column C was effective in enriching a fraction with nothofagin (fraction C2). Fraction C2, contrary to C3, had the highest IC_{50} (>10 mg/ml) for the inhibition of lipid peroxidation ($P<0.05$) and a value of 0.066 mg/ml for the $ABTS^{+\bullet}$ assay. Fraction C2 was subjected to the precipitation process as described earlier. The residue was applied to the reverse phase column D while the supernatant and filtrate were collected, pooled and evaporated to give ‘noto_lye’. The total polyphenol content of ‘noto_lye’ was 65%. Recognisable peaks of ‘noto_lye’ include 71.69% nothofagin, 4.76% aspalathin and 1.37% vitexin/ferulic acid.

This fraction was effective ($P<0.05$) as scavenger of $ABTS^{\bullet+}$ (IC_{50} value of 0.091 mg/ml), but not as inhibitor of lipid peroxidation (IC_{50} value of 1.505 mg/ml). 'Noto_lye' stimulated 2-AAF induced mutagenesis in TA98 at all the concentrations tested. Fraction D2 (Fig. 3.2) consisted of 99.03% nothofagin and 0.97% aspalathin (HPLC) (Addendum 3F). Purity was verified by LC-MS (Addendum 3G). Fraction D2 was evaluated as pure nothofagin and its biological activities reported in chapters 4 and 5.

Due to the small volumes isolated and the relatively large amounts necessary for the biological assays, not enough nothofagin was available for NMR analyses. The isolation of pure nothofagin was laborious and time-consuming. In order to obtain enough pure nothofagin for the biological assays (90 mg was needed to perform all the assays without repeats), two separate X3 fractions had to be purified as firstly the unfermented rooibos extracted was not particularly high in nothofagin content and secondly the yields on the nothofagin columns were low. The nothofagin yield for the C_{18} column was about 18%. This may however not be a true reflection of the efficiency of this column as the precipitation step only partially precipitated nothofagin; the 'noto_lye' still contained 71% nothofagin. However, as is the case with aspalathin, a considerable amount of the material loaded on the column was adsorbed onto the C_{18} material. The adsorbed material was removed during the regeneration process. The rinse solvents were pooled and evaluated on TLC, but no compound could be identified. Fractions D1 and D3 had very low mass yield and were insufficient to be used in the biological assays or analyses. Even pooling similar D1 and D3 fractions from consecutive C_{18} columns did not yield

a mass of more than 2 mg of which enough could be removed from the sides of the vessel for HPLC analysis.

Microsomal lipid peroxidation

The correlation coefficient (R^2) of the fit of the data points for the inhibition of lipid peroxidation to the model for a sigmoidal dose response curve with variable slope was higher than 0.99 for all the fractions (Table 3.3; Figure 3.3). R^2 for the extracts were above 0.98. Though not significantly different, fraction X3 had the highest potency (0.1098 mg/ml) of the XAD fractions, but this potency decreased with further fractionation. X2 was equipotent with an IC_{50} value ($P>0.05$) of 0.1308 mg/ml. The activity of fraction X2 was also displayed in fractions A3 and B3 with IC_{50} values of 0.2457 and 0.1137 mg/ml, in that order. Fraction C2 was the most ineffective ($P<0.05$) inhibitor of lipid peroxidation of all the extracts and fractions tested (>10 mg/ml).

$ABTS^{+\bullet}$ Assay

The correlation coefficient (R^2) of the fit of the data points for the scavenging of $ABTS^{+\bullet}$ to the model for a sigmoidal dose response curve with variable slope was higher than 0.98 for all the extracts and fractions, except fraction A3 (R^2= 0.9783) (Table 3.3; Figure 3.3). Fraction X2 (0.35 mg/ml) exhibited the most effective ($P<0.05$) ability to quench the radical of all the XAD fractions and this ability was increased ($P<0.05$) by purification to 0.025 (fraction A3) and 0.024 mg/ml (fraction B2). The potency of fraction B2 and B3 was not significantly different from that of fraction C3 (0.030 mg/ml) ($P>0.05$), which was to be expected as 63% of C3 consists of aspalathin.

Mutagenicity Assay

The aqueous and methanol extracts of unfermented rooibos exhibited a similar ($P>0.05$) ability to inhibit 2-AAF induced mutagenesis in TA98 at the highest (20 mg/ml) concentration tested (98 and 99%), at 10 and 5 mg/ml the methanol extract being the more efficient inhibitor ($P<0.05$) and at 1 mg/ml the aqueous extract showed a 19% inhibition versus the 7% of the methanol extract ($P<0.05$). The chloroform extract performed significantly weaker ($P<0.05$) than the other two extracts at the higher concentrations, changing from an inhibitory extract to one that stimulated 2-AAF induced mutagenesis at 5 and 1 mg/ml. Fractions X1 to X4 of the XAD-2 column were equally active ($P<0.05$) at the 20 mg/ml level, but Fraction X1 displayed the highest antimutagenic potential (27% inhibition) at a concentration of 1 mg/ml tested against TA98. C4 (54% total polyphenols)(Fig. 3.2), the last fraction off the Sephadex column used for nothofagin isolation, showed the highest antimutagenic activity ($P<0.05$) against TA98 of all the fractions of the different isolation columns (columns A to C) at the two highest concentrations tested, i.e. 99% (10 mg/ml) and 88% (5 mg/ml). At a concentration of 1 mg/ml, however, the percentage inhibition obtained was not significantly higher than many of the other fractions. Comparing the responses of the isolation columns A, B and C, no potent antimutagenic response was noted by any fraction at 1 mg/ml. At this concentration (1 mg/ml), the highest inhibitory response obtained for the XAD fractions was 27% (X1) and an equipotent ($P>0.05$) 28% for A2 of columns A to C.

The chloroform extract and fractions that stimulated the 2-AAF induced mutagenic response like X4 and X5 at 1 mg/ml, were not evaluated for direct,

co- or promutagenicity. The supernatant of the precipitation process of nothofagin, ‘noto_lye’ also caused a strong increase in the number of TA98 colonies.

The responses of most of the extracts and fractions were typically dose dependant inhibitions, i.e. a dilution in the concentration of the testing material also leads to a lower inhibitory response. As the fractionation of the X1 aliquot did not form part of this study, an enriched antimutagenic fraction has not yet been isolated.

DISCUSSION

Aspalathin and nothofagin, two of the major dihydrochalcones of rooibos, have been isolated to a purity of >95% following a process of antioxidant activity-guided fractionation. As neither of these compounds have a crystalline structure, the basic amorphous isolates are used as standards for HPLC, making quantification not completely reliable. An HPLC chromatogram however, still is a most useful tool for quantification as impurities in the range of detection would show. To the same extent LCMS and NMR are analysis tools with which impurities can be assessed and on the basis of the detectable impurities present, the purity of both the isolated aspalathin and nothofagin is set at >95%.

The polarity of the extraction solvent/eluant seems to determine the nature of the biological activity of the extract or fraction as the higher polyphenol content of the methanol extract indicated that the polarity index of solvents used for the purpose of isolation of the active compounds should be around 5. Fractions of a

high total polyphenol content and that yielded good antioxidant activity were eluted in 25 to 50% methanol/water solutions. Fractions extracted by solvents of higher polarity, generally had lower total polyphenol contents but were more potent inhibitors of 2-AAF induced mutagenesis.

Lipophilic extracts like chloroform and fractions such as X5 contained a small amount of phenolics and even displayed a stimulation of the mutagenic response at lower concentrations. The stimulation of mutagenic response by the X5 fraction can also possibly be linked to the preparation of the extract, a final elution of the XAD-2 column by chloroform:methanol:1:1. It is not known if the non-polar beads of Amberlite XAD-2 could be treated with chloroform. It is possible that by using this non-polar mixture as eluant, surface material from the beads themselves could have been stripped, causing the stimulation of response in lipid peroxidation as well as 2-AAF induced mutagenesis in TA98.

Flavonoids appear to have a synergistic effect on each other to enhance their activity in certain assays. Fraction B1 (15.77% aspalathin, 27.37% orientin and 23.42% isoorientin) and B3 (~99% aspalathin and 0.29% orientin) are equipotent to B2 (95.67% aspalathin, 2.99% orientin and 0.10% isoorientin) in scavenging $ABTS^{+\bullet}$ even though the contribution of each individual flavonoid varies. The IC_{50} values obtained by pure aspalathin, orientin and isoorientin in the $ABTS^{+\bullet}$ assay are 0.027, 0.089 and 0.086 mg/ml respectively (see Chapter 4). Despite the higher IC_{50} values for orientin and isoorientin, their combined effectiveness in scavenging the radical can be ascribed to a possible synergistic effect. Another possibility could be the presence of minor compounds with

potent activity. The high percentage inhibition of 2-AAF induced mutagenesis by fraction C4 could also be the result of the presence of yet unidentified flavonoids as none of the individual flavonoids detected in C4, i.e. 7.1% aspalathin, 0.36% orientin and 0.32 % hyperoside could be responsible for such antimutagenic activity.

The decrease of the high potency of X3 in the lipid peroxidation assay with further fractionation was surprising, as one would have expected an increase in potency as the fractions are enriched with antioxidants. Looking at the raw data, the inhibition of lipid peroxidation by fraction X3 dropped very steeply from an average of 84% to 10% over two consecutive concentrations, i.e. 0.1 and 0.05 mg/ml, respectively (data not shown). This could imply a high rate of reaction kinetics (steep hill slope), but more data points within this range are needed to substantiate this statement.

Inhibitory data against 2-AAF induced mutagenesis obtained by B3 (aspalathin; 16, 15 and 7% for solutions of 10, 5 and 1 mg/ml, respectively) compared well with that obtained for pure aspalathin in the mutagenicity assay against TA98: 27, 17 and 16% (data not shown), respectively, for original solutions used in the assay at similar concentrations. The IC_{50} values obtained by aspalathin in the antioxidant assays also corresponded well with those of fraction B3: 0.190 vs 0.1137 mg/ml for lipid peroxidation and 0.027 vs 0.023 mg/ml for $ABTS^{\bullet+}$, respectively.

The good correlation between the total polyphenol content of a fraction and its antioxidant potency, especially in the scavenging of $ABTS^{+\bullet}$, was to be expected. The Folin-Ciocalteu (F-C) (Total Phenolics) assay has for many years been used as a measure for the total phenolics in natural products, but since the basic mechanism is a redox reaction this method is considered to be another antioxidant method (Huang et al., 2005; Prior et al., 2005).

It is noticeable that fraction B3, pure aspalathin, contained a mere 78% total phenols. In the case of the determination of the total phenol content of fraction B3, the percentage total phenols (as g gallic acid equivalents/100 g) was calculated from the dilution series of fraction B3 in DMSO with absorbance readings between 0.2 and 0.8 after reaction with the F-C reagent. The total phenol content of pure aspalathin was determined as 72% by using the ratio between the gradients of the standard curves for aspalathin and gallic acid (data not shown). It is known that the F-C method can suffer from interfering substances, for instance sugars (Prior et al., 2005) and it is not known what effect a high pH will have on aspalathin in particular. It is known that flavonoid compounds are unstable in alkaline solution and may undergo a direct chemical oxidation (Koeppen, 1970). These factors may thus contribute to a lower determined value for aspalathin, emphasizing the fact that this assay should only be used as an index of the total phenol content since different phenols might also react would react in varying ways in this assay.

No evident relationship was found between the antimutagenic and antioxidant properties of various fractions from unfermented rooibos. Future research

should involve the fractionation of fraction X1 to identify the compound responsible for the potent antimutagenic properties displayed by this fraction.

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CHAPTER 4

ANTIOXIDANT ACTIVITY OF THE MAJOR FLAVONOIDS OF ROOIBOS (*Aspalathus linearis*)

ABSTRACT

The flavonoids of rooibos (*Aspalathus linearis*) are a unique combination that lends itself to comparative structure-activity studies. The most abundant flavonoids are the dihydrochalcones aspalathin and nothofagin. Their flavone analogues are orientin and isoorientin from the precursor aspalathin and vitexin and isovitexin from nothofagin. The flavone aglycones include luteolin and chrysoeriol. Four flavonols from rooibos, i.e. quercetin, isoquercitrin (quercetin-3-*O*-glucoside), hyperoside (quercetin-3-*O*-galactoside) and rutin (quercetin-3-*O*-rutinoside) as well as the flavanol (+)-catechin were additionally investigated. Their potential as flavonoid antioxidants was assessed in the Fe(II) induced lipid peroxidation assay, using rat liver microsomes as a model membrane system together with the $ABTS^{\bullet+}$ assay. The performance of epigallocatechin gallate (EGCG) in the same assays was used as the benchmark. Aspalathin, the dihydrochalcone unique to rooibos, was demonstrated to be a very efficient inhibitor of lipid peroxidation and scavenger of $ABTS^{\bullet+}$, while nothofagin was shown to be equipotent to aspalathin in the $ABTS^{\bullet+}$ assay, but had the lowest inhibitory effect (highest IC_{50} value) of all the flavonoids tested in the lipid peroxidation assay. These results confirmed the importance of the B ring catechol moiety as being the radical scavenging entity (chain breaking) and metal chelator (preventor) for dihydrochalcones as well. Orientin and isoorientin had lower IC_{50} values for both antioxidant assays used than either vitexin or

isovitexin, a further confirmation of the importance of the B ring catechol moiety. The two C8-glycosyl isomeric forms of the flavones, isovitexin and isoorientin, were both poor inhibitors in the lipid peroxidation assay, but in the $ABTS^{\bullet+}$ assay isoorientin was 13 times more effective than isovitexin, which could possibly be due to the more polar nature of isoorientin. Orientin and vitexin, both angular C8-glycosyl isomeric forms of the flavones, could possibly have lower IC_{50} values in the lipid peroxidation assay because of the strong possibility that the carbohydrate moiety of the compound attaches itself to the polar head of the membrane thereby enhancing its scavenging activity in the non-polar environment of the membrane. Methylation of the C3´-OH group of luteolin to form chrysoeriol decreased the IC_{50} values in both the antioxidant assays. The introduction of sugar moieties on the flavonol C3-OH reduced the efficacy of quercetin in both assays, rendering isoquercitrin and hyperoside the weakest inhibitors of the flavonols in the $ABTS^{\bullet+}$ and lipid peroxidation assays respectively. The order of increasing IC_{50} values amongst the monomeric rooibos flavonoid aglycone for both the lipid peroxidation and $ABTS^{\bullet+}$ assays, would be quercetin>(+)-catechin>chrysoeriol> luteolin. This order emphasizes the fact that the C3-OH is a more important structural/functionality requirement for antioxidant activity than a C4-keto group.

INTRODUCTION

Rooibos is an indigenous South African shrub *Aspalathus linearis* that is used to prepare a traditional herbal drink. Lately it is being promoted as a health drink as it contains no caffeine (Blommaert & Steenkamp, 1978), has a low tannin content (Morton, 1983) and exhibits health properties (Snyckers and Salemi, 1974;

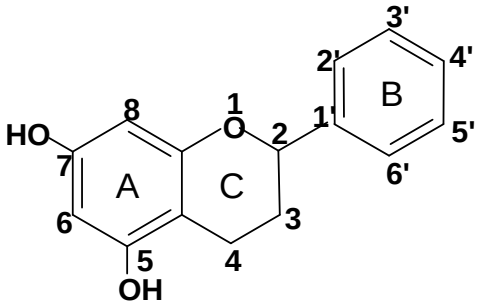
Joubert and Ferreira, 1996; Marnewick et al., 2000; Standley et al., 2001; Van der Merwe et al., 2006). The health properties of plants are generally attributed to their flavonoids and their antioxidative properties (Bors et al., 1997; Rietveld et al., 2003). Rooibos contains a selection of structurally related flavonoids that can be divided into four groups, i.e. dihydrochalcones, flavones, flavonol and flavanols (Koeppen et al., 1962; Koeppen, 1963; Rabe et al., 1994; Ferreira et al., 1995; Bramati et al., 2002). This unique combination of flavonoids lends itself to comparative structure-activity studies.

The most abundant flavonoids are the aspalathin and nothofagin (Joubert, 1996), described as two C-C linked β-hydroxy-dihydrochalcone glycosides, and can constitute up to 12% and 1.3% of the dry unfermented ('green') rooibos respectively (Schulz et al., 2000). Aspalathin is unique to rooibos (Koeppen and Roux, 1965) whereas to date nothofagin is found only in rooibos and *Nothofagus fusca* (Ferreira et al., 1995). Aspalathin is a precursor to the (S)- and (R)-eriodictyol-6-C-β-D-glucopyranoside flavanones dihydro-isoorientin and dihydro-orientin (Ferreira et al., 1995). It is possible, though it has not yet been shown, that a further conversion, presumably by oxidation, could lead to the formation of the flavones, orientin and isooorientin. Nothofagin, vitexin and isovitexin are the 3'-dehydroxy analogues of aspalathin, orientin and isoorientin, respectively. Other rooibos flavonoids include rutin (quercetin-3-*O*-rutinoside) and isoquercitrin (3-*O*-*ß*-*D*-glucopyranoside) (Koeppen et al., 1962), luteolin and quercetin (Snyckers and Salemi, 1974), chrysoeriol, vitexin, isovitexin (Rabe et al., 1994), (+)-catechin, (+)-pinitol, procyanidin B3, 5,7-dihydroxy-6-C-β-D-glucopyranosyl-chromone (Ferreira et al., 1995), hyperoside (quercetin-3-*O*-galactoside) and

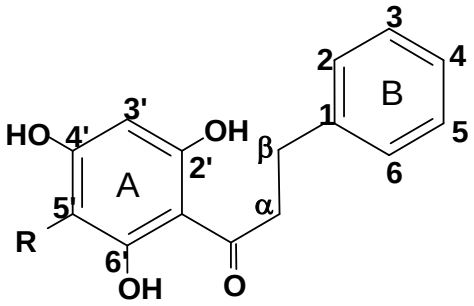
traces of the flavanones dihydroorientin and dihydro-isoorientin (Bramati et al., 2002), luteolin-7-glucoside (Kazuno et al., 2005) and aspalalinin (Shimamura et al., 2006). The structures of the major flavonoids of rooibos are shown in Table 4.1.

"Fermentation", i.e. chemical oxidation, of rooibos affects its antioxidant and antimutagenic properties. It has been shown that the aqueous soluble solids of unfermented, semi-fermented and fermented rooibos have stronger 2,2'-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging activity than that of black and oolong teas, but weaker than green tea (Von Gadow et al., 1997). Processing of rooibos also decreases its antioxidant activity with regards to the superoxide and DPPH radical scavenging ability, while results regarding processing and its effect on antimutagenicity in the *Salmonella typhimurium* assay dependant on sample size and sampling (Van der Merwe et al., 2006). Standley et al. (2001) concluded that fermented rooibos offered less protection than unfermented rooibos against 2-AAF, while Van der Merwe et al. (2006) showed that fermented rooibos was a more effective inhibitor of 2-AAF as well as AFB_1 induced mutagenesis. The activity of several of the rooibos flavonoids has been studied in various antioxidant assays. The radical scavenging activity of catechin, quercetin, isoquercitrin, rutin and luteolin were compared using the 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) assay (Re et al., 1999; Lien et al., 1999, Plumb et al., 1999), whereas Okawa et al. (2001) evaluated the activity of isovitexin, quercetin and rutin. Joubert et al. (2004) included the rooibos flavonoids aspalathin, orientin, isoorientin, luteolin, isoquercitrin, (+)-catechin, rutin, vitexin and chrysoeriol in their assessment of the

Table 4.1. Table showing structural differences amongst flavonoids as well as their metal chelating ability



Flavonoid



Dihydrochalcone

	C_2=C_3	C_3	C_4 C=O	C_6	C_8	B ring substitution	Bathochromic shift in peak (320-420 nm) +Fe^{2+} (nm)	Bathochromic shift in peak (320-420 nm) +EDTA (% recovery)
Dihydrochalcone								
Aspalathin						3, 4-dihydroxy	0	0
Nothofagin						4-hydroxy	0	0
Flavone								
Luteolin	+	-	+	-	-	3´, 4´-dihydroxy	26	93
Chrysoeriol	+	-	+	-	-	4´-hydroxy-3´-methoxy	0	0
Orientin	+	-	+	-	CG	3´, 4´-dihydroxy	27	96
Isoorientin	+	-	+	CG	-	3´, 4´-dihydroxy	25	95
Vitexin	+	-	+	-	CG	4´-hydroxy	0	0
Isovitexin	+	-	+	CG	-	4´-hydroxy	0	0
Flavanol								
(+)-Catechin	-	OH	-	-	-	3´, 4´-dihydroxy	0	0
Epigallocatechin gallate[#]	-	Gallate	-	-	-	3´,4´,5´-trihydroxy	0	0
Flavonol								
Quercetin	+	OH	+	-	-	3´, 4´-dihydroxy	46	71
Isoquercitrin	+	OG	+	-	-	3´, 4´-dihydroxy	19	92
Hyperoside	+	OGa	+	-	-	3´, 4´-dihydroxy	5	89
Rutin	+	ORu	+	-	-	3´, 4´-dihydroxy	23	98

ORu : O-rutinosyl; OG : O-glucopyranosyl; OGa : *O*-galactosyl; CG or R : C-glucopyranosyl; 0 : no result obtained.
[#]Green tea flavonoid used as reference.

DPPH and superoxide anion radical scavenging activities. Inhibition of lipid peroxidation in rat liver microsomes by (+)-catechin, luteolin, orientin, isoorientin, vitexin, rutin, hyperoside and quercetin has also been reported (Robak et al., 1988; Mora et al., 1990; Cos et al., 2001; Yang et al., 2001; Heijnen et al., 2002).

Two assays were selected for the present study to evaluate the antioxidant activity of the major rooibos flavonoids as antioxidants since each may respond in a different manner to different radical or oxidant sources (Frankel and Meyer, 2000; Aruoma, 2003; Prior et al., 2005; Huang et al., 2005). The microsomal lipid peroxidation assay, mimicking a lipophilic *in vivo* system, was selected to evaluate the ability of the antioxidant to protect the membraneous environment with respect to the generation of reactive oxygen species (Terao and Piskula, 1999; Webb and Ebeler, 2004). On the basis of the chemical reactions involved, this assay is regarded as a hydrogen atom transfer (HAT) reaction, since the hydrogen atom donating capacity and subsequent radical chain breaking ability is being evaluated in this method (Huang et al., 2005; Prior et al., 2005). An oxidative attack on the acyl chains of the membrane lipids would lead to the formation of radicals in the hydrophobic core of the bilayer. Their efficacy of the flavonoids in this assay would depend on their ability to access the hydrophobic core to neutralise the generated radicals. This in turn would depend on the structural conformation and hydrophobicity of the flavonoids (Cholbi et al., 1991; Williamson et al., 2000; Anselmi et al., 2004; Manach et al., 2004). On the other hand, the flavonoids could also act as preventative antioxidants by chelating the metal ions that initiate lipid peroxidation (Kandaswami and Middleton, 1994; Terao and Piskula, 1999; Pietta et al., 2000; Heim et al., 2002; Liu, 2005).

The second assay used was the $ABTS^{+\bullet}$ assay since this method has been employed extensively to assess the antioxidant activity of flavonoids in a hydrophilic environment (Rice-Evans et al., 1996; Plumb et al., 1999). Huang et al. (2005) as well as Prior et al. (2005) consider this process as a typical electron transfer (ET) based reaction since the radical cation abstracts an electron from the antioxidant. However, it might be difficult to distinguish mechanistically between HAT and ET reactions (Prior et al., 2005). Although $ABTS^{+\bullet}$, as $DPPH^{\bullet}$, is not a physiologically relevant radical, assays using these radicals are useful in providing a ranking order of antioxidants (Van den Berg et al., 1999; Okawa et al., 2001; Miliauskas et al., 2004).

Epigallocatechin gallate (EGCG), the most abundant and most active antioxidant of Japanese green tea (*Camellia sinensis*) (Hu and Kitts, 2001; Frei and Higdon, 2003) was used as reference flavonoid.

MATERIALS AND METHODS

Reagents

Aspalathin and nothofagin were isolated from unfermented rooibos to a purity of >95% at the PROMEC Unit. HPLC grade flavonoids, i.e. chrysoeriol, orientin, isoorientin (as homoorientin), vitexin, isovitexin and isoquercitrin (as quercetin-3-*O*-glucopyranoside) were obtained from Extrasynthese (Genay, France). Luteolin, quercetin (as quercetin dihydrate), rutin (>95% pure), (+)-catechin (>98% pure) and epigallocatechin gallate (EGCG; >95%) were purchased from Sigma-Aldrich S.A. Solvents and chemicals such as 2,2´-azinobis-(3-ethylbenzo-

thiazoline-6-sulfonic acid) (ABTS); 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich S.A. All solvents and reagents were of analytical grade and all chemicals were used as purchased. The chemical structures of the various dihydrochalcones and flavonoids used in this study are shown in Table 4.1.

Inhibition of microsomal lipid peroxidation (TBARS)

Rat liver microsomes were prepared from male Fisher (F344) rats (200-300 g) as described by Shen et al. (1994). Lipid peroxidation was performed according to the method of Yen and Hsieh (1998) with slight modifications. The microsomes were diluted with a 0.2 M potassium phosphate buffer (pH 7.4) to a concentration of 1 mg protein/ml. Lipid peroxidation was induced by adding 5 µM ferrous sulphate (200 µl) into a total volume of 1 ml, containing the different flavonoids dissolved in 100 µl DMSO and 700 µl microsomal suspension in buffer. The reaction mixture was incubated for 1 hour at 37°C. For each sample a sample blank was included as the compounds themselves could possibly absorb at 532 nm; any reading was subtracted from the actual sample readings. A positive control using 100 µl DMSO as well as reaction blank with buffer without microsomes to be subtracted from the positive control was included. The reaction was terminated by adding 2 ml of a cold 10% (m/v) trichloroacetic acid (TCA) solution containing butylated hydroxytoluene (BHT, 0.01% m/v) and ethylenediaminetetraacetic acid (EDTA, 0.1% m/v). Samples were centrifuged, 2 ml of the supernatant mixed with 2 ml of a 0.67% (m/v) thiobarbituric acid (TBA) solution, heated for 20 min at 90°C and the thiobarbituric acid reactive substances (TBARS) measured at 532 nm. The percentage inhibition of TBARS

formation relative to the positive control was calculated by $\frac{(A_{control} - A_{sample})}{A_{control}} \times 100$, where $A_{control}$ and A_{sample} refer to the corrected absorption readings for the positive control and sample, respectively. The assay was conducted in triplicate. The IC_{50} values were calculated by using GraphPad Software (Prism Version 5.00 for Windows) as described in Addendum 3A.

$ABTS^{\bullet+}$ Decolourization Assay ($ABTS$ assay$^{\bullet+}$)

The 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) assay was performed as described by Re et al. (1999) with minor modifications. The radical cation was generated by mixing ABTS (7 mM) and potassium persulphate (2.45 mM) in water and left in the dark for 12-16 h prior to use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm (30°C). The diluted $ABTS^{\bullet+}$ solution (1 ml) was added to 50 µl of the flavonoid solution in DMSO, heated at 30°C for 4 minutes and the absorbance measured at 734 nm. Sample as well as reaction blanks were included in each assay and determinations at each concentration were done in triplicate. The percentage inhibition of the radical cation was calculated as described above and expressed as an IC_{50} value, calculated by using GraphPad Software (Prism Version 5.00 for Windows).

Uv-vis spectroscopy of flavonoid metal complexes

Bands appearing after addition of iron and disappearing after addition of the chelators EDTA can be ascribed to the formation of a complex between the phenolic functional groups of the compounds and iron (Mira et al., 2002). The

iron chelating potential of flavonoids was compared according to the method described by Moridani et al. (2003). A 2.5 mM stock solution of each flavonoid was prepared in DMSO. The flavonoid (20 µl) was added to a cuvette containing 10 mM potassium phosphate buffer (pH 7.4) (1.98 ml) to give a final concentration of 25 µM flavonoid in the reaction volume. The absorption spectrum was recorded between 200-700 nm. Ferrous sulphate was then added to the cuvette (final concentration of 50 µM in reaction volume) and scanned after 5 min. The reversibility of the flavonoid:Fe^{2+} ion complex was evaluated by the addition of 1.25 mM EDTA (in final reaction volume) to the cuvette 5 minutes prior to scanning.

Statistical analyses

Compounds were divided into three groups according to their performance in each assay. Within each group the F-test for testing differences in fit between two non-linear models was used. These tests were done on all pairs of all compounds and P-values were corrected using the Bonferonni post-hoc correction. A significance level of 95 % ($P<0.05$) was used as guideline to determine differences between models.

RESULTS

Inhibition of Fe(II) induced lipid peroxidation

The IC_{50} values, calculated as described above, are summarized in Table 4.2 along with the lower and upper values of the 95% confidence limits of the calculated IC_{50} value as well as the coefficient of correlation (R^2) between the model used and the data points of flavonoid responses.

Table 4.2. Results, expressed as IC_{50} values, of the lipid peroxidation (TBARS) antioxidant assay.

	Lipid peroxidation	
	IC_{50} value (µM)	**R^2**
Good inhibitors		
Aspalathin	50 (45.6; 55.3) **a**	0.9974
(+)-Catechin	52 (45.5; 59.2) **a**	0.9958
Epigallocatechin gallate[#]	21 (20.5; 21.4) **c**	0.9998
Chrysoeriol	67 (37.3; 120.4) **a**	0.9831
Quercetin	18 (16.9; 18.1) **b**	0.9974
Average inhibitors		
Luteolin	199 (166.6; 237.0) **d**	0.9943
Orientin	158 (117.6; 212.1) **de**	0.9896
Rutin	174 (156.4; 194.2) **de**	0.9962
Isoquercitrin	105 (99.3; 112.0) **f**	0.9965
Hyperoside	273 (257.5; 288.5) **g**	0.9989
Poor inhibitors		
Isoorientin	724 (386.7; 1355) **h**	0.9894
Vitexin	1113 (1048; 1183) **h**	0.9914
Isovitexin	1648 (1485; 1830) **i**	0.9910
Nothofagin	2506 (1378; 4557) **i**	0.9922

IC_{50} values, calculated from 3 replicates per treatment, are shown with lower and upper 95% confidence levels, respectively. Values in column followed by the same letter do not differ significantly ($P>0.05$). IC_{50} value for Trolox in lipid peroxidation assay is 75.7 µM. Concentration indicated as per final reaction volume.

[#]Green tea flavonoid used as reference.

In the rat liver microsomal lipid peroxidation assay quercetin (18 μM) and EGCG (21 μM) displayed the lowest IC_{50} values ($P<0.05$) followed by aspalathin (50 μM) and (+)-catechin (52 μM) (Table 4.2). A significant ($P<0.05$) decrease in efficiency was, however, noted for orientin (158 μM) and isoorientin (724 μM), the oxidised products of aspalathin. Nothofagin (2506 μM) had the highest IC_{50} value comparable to that of isovitexin (1689 μM) ($P>0.05$). Vitexin (1113 μM), however, had a significantly lower IC_{50} value than that of nothofagin (2506 μM) and isovitexin (1689 μM) ($P<0.05$). Isoorientin (724 μM) was as effective as vitexin ($P>0.05$). Chrysoeriol (67 μM) exhibited a similar ($P>0.05$) inhibitory effect to aspalathin (50 μM) and (+)-catechin (52 μM) while the demethylated flavone luteolin (199 μM) showed less protection ($P<0.05$). The glycosylated flavonols hyperoside (273 μM), isoquercitrin (105 μM) and rutin (174 μM) were substantially less effective ($P<0.05$) than their aglycone, quercetin (18 μM). The potency of the rooibos flavonoids and EGCG as inhibitors of microsomal lipid peroxidation, in decreasing order, is quercetin > EGCG > aspalathin ≈ (+)-catechin ≈ chrysoeriol ≥ isoquercitrin > orientin ≥ luteolin ≥ rutin > hyperoside >> isoorientin ≈ vitexin > isovitexin ≥ nothofagin.

Radical scavenging $ABTS^{\bullet+}$ Decolourization Assay

The radical scavenging activity of aspalathin (IC_{50} = 3.24 μM) was not significantly ($P>0.05$) different from that of quercetin (3.55 μM) and EGCG (3.52 μM), while the difference between the activity of nothofagin (4.69 μM) and EGCG (3.52 μM) is also not significantly different (Table 4.3). The post-hoc Bonferroni test indicated that there is a significant difference ($P<0.05$) between the activity of nothofagin (4.69 μM) and that of catechin (5.40 μM). The flavone analogues of

Table 4.3. Results, expressed as IC_{50} values, of the $ABTS^{\bullet+}$ antioxidant assay.

	ABTS	
	IC_{50} value (µM)	**R^2**
Good radical scavengers		
Aspalathin	3.24 (3.02; 3.47) **a**	0.9991
Nothofagin	4.69 (3.92; 5.63) **b**	0.9910
Epigallocatechin gallate[#]	3.52 (3.01; 4.11) **ab**	0.9941
Quercetin	3.55 (3.27; 3.85) **a**	0.9995
Chrysoeriol	8.87 (8.17; 9.63) **d**	0.9983
(+)-Catechin	5.40 (4.35; 6.69) **c**	0.9985
Hyperoside	6.27 (5.79; 6.79) **c**	0.9936
Average radical scavengers		
Luteolin	10.82 (10.16; 11.52) **e**	0.9965
Orientin	11.43 (10.99; 11.88) **f**	0.9993
Isoorientin	11.14 (10.54; 11.78) **ef**	0.9986
Rutin	11.01 (9.82; 12.36) **ef**	0.9971
Isoquercitrin	19.23 (17.21; 21.49) **g**	0.9998
Poor radical scavengers		
Isovitexin	145.4 (127.4; 166.0) **h**	0.9978
Vitexin	287.2 (242.4; 340.2) **i**	0.9958

IC_{50} values, calculated from 3 replicates per treatment, are shown with lower and upper 95% confidence levels, respectively. Values in column followed by the same letter do not differ significantly ($P>0.05$). IC_{50} value for Trolox in ABTS assay is 12 µM. Concentration indicated as per final reaction volume.
[#]Green tea flavonoid used as reference.

aspalathin (3.24 μM), viz. isoorientin (11.14 μM) and orientin (11.43 μM), exhibited higher IC_{50} values than the dihydrochalcone but were equipotent ($P>0.05$) to each other, their aglycone luteolin (10.82 μM) as well as chrysoeriol (8.87 μM). The flavone analogues of nothofagin, i.e. isovitexin (145.4 μM) and vitexin (287.2 μM) exhibited significantly ($P<0.05$) higher IC_{50} values than their dihydrochalcone. Vitexin followed by isovitexin were the weakest radical scavengers of the flavonoids tested. The maximum plateau of their sigmoidal dose response curve was at about 50% and 40% inhibition respectively (data not shown). Hyperoside (6.27 μM) was less effective than quercetin (3.55 μM), but more effective than isoquercitrin (19.23 μM) and rutin (11.01 μM) ($P<0.05$). (+)-Catechin (5.40 μM) was equipotent ($P>0.05$) to hyperoside (6.27 μM). The potency of the rooibos flavonoids and EGCG in scavenging the $ABTS^{\bullet+}$ cation assay, in a descending order, is: aspalathin ≈ quercetin ≈ EGCG > nothofagin > (+)-catechin ≈ hyperoside > chrysoeriol > luteolin ≥ isoorientin ≥ rutin ≥ orientin > isoquercitrin >> isovitexin >> vitexin.

Iron Chelation

No band I shifts (320-420 nm) were observed for aspalathin and nothofagin and none of the compounds evaluated could produce a band II shift in the A ring region (250-320 nm). The complexation of Fe(II) by quercetin resulted in a 46 nm band shift in the band I region while that of rutin, isoquercitrin and hyperoside caused shifts of 23, 19 and 5 nm, respectively (Table 4.1). The chelation of Fe(II) by luteolin, orientin and isoorientin resulted in band shifts in this region from 25 to 27 nm. Addition of EDTA was able to reverse more than 90% of these shifts, except in the case of quercetin where only 71% of the compound was recovered

from the complex. (+)-Catechin, EGCG, chrysoeriol, vitexin or isovitexin were not able to complex Fe(II) with the B ring.

DISCUSSION

Aspalathin, the dihydrochalcone unique to rooibos, was a very efficient inhibitor of both lipid peroxidation and $ABTS^{\bullet+}$ within the context of the rooibos flavonoids. The inhibitory response, however, of its 4-dehydroxylated counterpart, nothofagin, clearly illustrated the necessity of using more than one method to evaluate the antioxidant activity of a substance due to the finding that it was equipotent to aspalathin in scavenging $ABTS^{\bullet+}$, but was the least efficient of all the flavonoids tested in inhibiting lipid peroxidation. The slightly higher IC_{50} value of nothofagin to that of aspalathin in the $ABTS^{\bullet+}$ assay can be attributed to the absence of the one hydroxyl group in the B-ring since the activity of one of the hydroxyl groups of the catechol moiety in ring B is enhanced by the electron donating effect of the adjacent hydroxyl group (Haenen et al., 1997; Heijnen et al., 2001).

Rezk et al. (2002) studied phloretin, a dihydrochalcone with a C4-hydroxyl group on the B ring and phloridzin, the C6′-glucoside of phloretin, in peroxynitrite scavenging and the inhibition of rat microsomal lipid peroxidation. The activity of the hydroxyl group at position 3 was enhanced by the electron donating effect of the hydroxyl groups at positions 5 and 7. A carbonyl group on C4 additionally increases the antioxidant activity of phloretin (Rezk et al., 2002). Neither aspalathin nor nothofagin has a C3-OH group, but both are 2′,4′,6′-trihydroxylated in Ring A (4′,6′-dihydroxylation of dihydrochalcones is similar in

pattern to 5,7-dihydroxylation of flavonoids since the latter do not have a 2′-OH group as it has been incorporated into the pyran ring C). Rezk et al. (2002) proposed that the dihydrochalcone radical that is formed after hydrogen abstraction may be stabilised in a way similar to the strong activity of 2,6-dihydroxyacetophenone, i.e. by a keto-enol tautomeric transformation between the carbonyl group and the R-methylene. Both aspalathin and nothofagin has a hydroxyl group at the 2′-position of the A ring, an essential pharmacophore for the radical scavenging potential of a dihydrochalcone (Nakamura et al., 2003).

Another possible explanation for the scavenging activities of the two rooibos dihydrochalcones to be considered, may be found in the hypothesis put forward by Mathiesen et al . (1997). In their NMR studies on two dihydrochalcones viz. myrigalone B (2′,6′-dihydroxy-4′-methoxy-3′,5′-dimethyl-dihydrochalcone) and angoletin (2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethyl-dihydrochalcone) the spectra showed that the A ring which is an active radical scavenger moiety of the dihydrochalcone is orthogonal to the carbonyl group in the side chain. Abstraction of one of the *ortho* phenolic hydrogen atoms during radical scavenging, the thereby formed highly active phenoxy radical may adopt a coplanar conformation forming a strong intramolecular hydrogen bond between the remaining phenolic hydrogen and the carbonyl group. Mathiesen et al. (1997) concluded that substances with an orthogonal conformation able to form intramolecular hydrogen bonds by loss of a phenolic hydrogen, would be $DPPH^{\bullet}$ scavengers, while compounds lacking these properties would be inactive.

The ability for these systems to chelate metal ions could also be responsible for the low IC_{50} value of aspalathin in the lipid peroxidation assay due to the fact that aspalathin possesses two of the three possible binding sites for metal ions by flavonoids listed by Yuting et al. (1990) and Cholbi et al. (1991), i.e. the catechol moiety in the B ring and between the keto group and C2′-OH in ring A. In physiological liquids, the Fe(II) cation would be present as an aquacomplex with a variable number of coordinating water molecules (Leopoldini et al., 2006). Aspalathin, having a polar nature, will also be present in the aqueous phase. The binding of the transition metal ions to the biological target is a prerequisite for the $^{\bullet}OH$ radical mediated cell damage (Samuni et al., 1983). Chelation of Fe(II), the initiating agent of the assay, would prevent the formation of hydroxyl radicals and the subsequent progress of lipid oxidation will be consequently retarded. Moridani et al. (2003) in fact determined the ability of a flavonoid to complex Fe^{2+} ions by monitoring bathochromic shifts related to band I or band II shifts. In the present study using Moridani's method, aspalathin was however unable to complex Fe(II) ion, or was insensitive to the assay used. This finding suggests that the efficacy of aspalathin in the lipid peroxidation assay can therefore not be explained by the chelation ability although its role as preventor of oxidation by metal chelation seems structurally plausible. The radical scavenging activity of flavonoids still prevails after metal complexation processes (Laughton et al., 1989; Hanasaki et al., 1994; Hider et al., 2001). Aspalathin, associated with the polar heads of the membrane, could thus still be able to scavenge any radicals that might have been formed.

This hypothesis would also explain the inability of nothofagin to inhibit/prevent lipid peroxidation. It was initially expected that the less ring B hydroxylated nothofagin would enable it to perform better in the more lipophilic environment of the lipid peroxidation assay. Nothofagin should have had a higher affinity for the hydrophobic core of the membrane, enabling it to scavenge radicals between the acyl chains of the membrane. This, however, was not found to be the case as it was determined that nothofagin had the highest IC_{50} value of all the flavonoids tested. Nothofagin has, however, only one site for metal ion chelation and without the presence of the B ring catechol moiety, it is possible that nothofagin cannot retain lipid peroxidation by its scavenging properties only.

Whether a dihydrochalcone will penetrate into a biological membrane or not is still not clear. If the rooibos dihydrochalcones, similar to the compounds studied by Mathiesen et al. (1997), are able to form phenoxy radicals that are able to adopt a coplanar structure, then membrane penetration would be facilitated. According to polarities, aspalathin should be scavenging radicals amongst the more polar heads of the phospholipids and nothofagin between the less polar acyl chains. Whether this assumption regarding molecular orientation is valid or not, will no doubt be clarified by further NMR-based studies.

The flavone analogues derived from aspalathin, i.e. orientin and isoorientin, had lower IC_{50} values for both antioxidant assays employed than the corresponding flavone analogues derived from nothofagin, i.e. vitexin and isovitexin. This observation further emphasizes the importance of the ring B catechol moiety. The IC_{50} values of both orientin and isoorientin, were higher in both the lipid

peroxidation and $ABTS^{\bullet+}$ assays than that of their precursor, with isoorientin being a far less efficient inhibitor of lipid peroxidation than orientin (Table 4.3). Both were able to chelate and release Fe(II) in the presence of EDTA to the same extent, contrary vitexin and isovitexin. The two C6-glycosyl isomeric forms of the flavones, isovitexin and isoorientin, were both poor inhibitors in the lipid peroxidation assay, but in the $ABTS^{\bullet+}$ assay isoorientin was 13 times more effective than isovitexin, possibly due to the more polar nature of isoorientin. Orientin had the lowest IC_{50} value of the glycosylated flavones, similar to those of the aglycone luteolin in both antioxidant assays, suggesting that the angular C8-glycoside, orientin, promoted an increase in the activity while the linear C6-glycoside, isoorientin, decreased activity. Mora et al. (1990) also reported an enhanced protection from orientin when compared to isoorientin in the microsomal lipid peroxidation assay, while Mun'im et al. (2003) reported strong activities from orientin and isoorientin towards $DPPH^{\bullet}$ and linoleic acid peroxidation, as well as against bactericidal action of peroxyl radical. Both flavones had an IC_{50} value of 9.5 µM and a MIC of 62 µM, respectively, for the $DPPH^{\bullet}$ and bactericidal action of peroxyl radical assays, whereas the IC_{50} value for vitexin was >60 µM and the MIC value 250 µM for these assays, respectively.

The degree to which the polarity of these flavonoids is affected by the relative position of the glucose moiety on the A ring, is still not known. However, both orientin and isoorientin are known to be polar molecules which will be expected to be associated with the aqueous phase of the membranes. However, in the case of orientin which has the glucose moiety on C8 hydrogen bonds are more likely to form between the hydroxyl groups of the sugar moiety and BC rings of

the flavonoid, resulting in a more rigid and stable conformation of the molecule. The flavones are however in general known to be planar (Harborne and Baxter, 1999) and with orientin with the *O*-glucopyranosyl group at C8 and thus having a more rigid conformation, it can penetrate the membrane bilayer more easily and thereby scavenge the formed radicals. However, in the case of isoorientin, in which the *O*-glucopyranosyl group I attached to C6, the generated alternative structural geometry may cause a sufficient degree of steric hindrance suppressing the flavone from membrane penetration. The same argument can be used to explain the relative performances of vitexin vs that of isovitexin as inhibitor of lipid peroxidation.

Flavonoids are also known to anchor themselves through chemical bonds to the polar head of phospholipids, forming reversible physicochemical complexes (Bombardelli and Spetta, 1991). As both the C8-glycosylated flavones have lower IC_{50} values, it is possible that the carbohydrate moiety of the compound attaches itself to the polar head of the membrane, thereby enabling a prolonged contact between the rest of the molecule and a subsequent enhanced antioxidant activity in the non-polar environment of the membrane (Anselmi et al., 2004; Erlejman et al., 2004).

The radical scavenging ability of flavonoids is also affected by the position or attachment of the sugar moiety as is demonstrated by orientin being a more efficient scavenger of the superoxide radical than isoorientin (Joubert et al., 2004). However, in scavenging the $DPPH^{\bullet}$ the two isomers were almost equipotent (Joubert et al., 2004), as was the case in the scavenging of the

$ABTS^{\bullet+}$ radical in this study. The higher efficacy of the C6-glycosylated isovitexin to that of the C8-glycosylated vitexin in scavenging $ABTS^{\bullet+}$ can not yet be explained.

Luteolin, the flavone aglycone, showed average antioxidant activity in both assays. Addition of a glucoside on C6 or C8 to form isoorientin or orientin did not affect the radical scavenging activity of luteolin much, though the inhibiting potential of orientin was significantly higher. However, in the case of orientin the antiperoxidative activity was increased, though not significantly, but in the case of isoorientin it was significantly decreased, possibly due to the presence of the sugar moiety effecting a favourable orientation for penetration of the membrane.

The flavonol quercetin had the lowest IC_{50} values of all the flavonoids investigated in both the lipid peroxidation and $ABTS^{\bullet+}$ assays. Its flavone counterpart luteolin was far less effective in both assays. Both of these molecules are aglyconic incorporating B-ring catechol moieties; planar due to the C2-C3 double bond and contain a C4-keto functional group. The structural difference between the two molecules in question is the additional C3-OH group present in quercetin which affects the polarity of the molecule and subsequently the ultimate distribution of the flavonoid in the membrane. The significance of this is that the most apolar flavone will be located deeper in the membrane towards the hydrophobic core of the bilayer while, on the other hand, increasing polarity resulting form an increasing number of hydroxyl groups in the molecule provides these flavonoids with a higher propensity in associating more with the aqueous phase (Scheidt et al., 2004). In the C ring the hydroxyl group at position 3 is the

only reactive one and its activity will be enhanced by the electron donating effect of the two hydroxyl groups at positions 5 and 7 as well as a carbonyl group at C4 (Rezk et al., 2002). The presence of a C3-OH in quercetin but not in luteolin will therefore explain the lower IC_{50} value of quercetin in the $ABTS^{\bullet+}$ assay.

The structural moiety comprising the C4 carbonyl and the C3 hydroxy group may be strongly responsible for one of the metal chelation sites in quercetin (Yuting et al., 1990; Cholbi et al., 1991), enabling quercetin to prevent the initiation of lipid peroxidation. Quercetin is also more hydroxylated than luteolin, making quercetin the more polar of the two compounds. Pawlikowska-Pawlęga et al. (2003) showed that quercetin only influenced the polar region of the bilayer of human erythrocyte membranes, protecting the surface against peroxidation and leaving the hydrophobic core of the membrane unchanged.

Introduction of sugar moieties on the flavonol C3-OH had the effect of increasing the IC_{50} value of quercetin in the $ABTS^{\bullet+}$ assay and it was found that the activity of hyperoside (C3-*O*-galactose) was between that of quercetin and rutin (C3-*O*-rutinose) with isoquercitrin (C3-*O*-glucose) being the least efficient radical scavenger of the flavonols. This ranking was not influenced by the moiety being a monosaccharide or a disaccharide. The efficiency of quercetin in the lipid peroxidation assay was reduced significantly with the addition of glucose (isoquercitrin), rutinose (rutin) and galactose (hyperoside), in this order. Hyperoside was a less efficient metal chelator than rutin and isoquercitrin, the latter being equally effective. The presence of a sugar moiety did however reduce the antiperoxidative activity of adjacent hydroxyl groups due to the

additional steric hindrance such groups would generate (Ratty and Das, 1988; Cholbi et al., 1991; Mora et al., 1990). Saija et al. (1995) showed that the glycoside group of rutin lowered its liposolubility and hampered its penetration into a model membrane and subsequent incorporation between the acyl chains of lipids.

When comparing the monomeric rooibos flavonoid aglycones for antioxidant activity, the order of increasing IC_{50} values for both the lipid peroxidation and $ABTS^{\bullet+}$ assays would be quercetin>(+)-catechin>chrysoeriol>luteolin. This order emphasizes the fact that the C3-OH group is a more important structural requirement for antioxidant activity than a C4-keto group. In liposomes, the C3-OH of quercetin enhanced the planarity of the molecule, giving higher rigidity to the ring and holding the A and C rings in a more coplanar position (Silva et al. 2002).

Methylation of the C3´-OH group in luteolin to form chrysoeriol significantly decreased the IC_{50} values in both the antioxidant assays. Luteolin, but not chrysoeriol, also responded in the metal chelation assay. The increased efficiency of chrysoeriol in the microsomal assay could, despite the compromised B ring catechol moiety, be attributed to its decreased hydrophilicity, enabling chrysoeriol to act as an antioxidant in a more lipophilic environment. Methoxy groups are known to enhance the lipophilicity and membrane partitioning ability of flavonoids (Heim et al., 2002). 3'-O-methyl catechin and 4'-O-methyl catechin, lacking metal chelating structures was, however, less effective than (+)-catechin in protecting against low-density lipoprotein oxidation (Cren-Olivé et al., 2003).

Chrysoeriol could also act as a radical scavenger for a longer time, since a phenoxyl radical can be stabilized by the electron donating ability of a methoxy group, resulting in a more efficient performance as antioxidant (Danilewicz, 2003). Joubert et al (2004), however, attributed the poor performance of vitexin and chrysoeriol as superoxide scavengers in an aqueous environment, to the presence of only one hydroxyl group on the B ring.

Hydrogenation of the double bond in the C ring, as was found by Mora et al. (1990), as well as the absence of the C4-keto group, significantly decreased the antiperoxidative and radical scavenging effects of quercetin. The 2,3 double bond in the C ring in conjunction with the C4-keto group improves electron delocalisation, stabilising the radical (Bors et al., 1990). However, in an investigation that included luteolin, quercetin and (+)-catechin, Cholbi et al. (1991) concluded that the C2-C3 double bond in the C ring and the C4-keto group is not essential to inhibit lipid peroxidation. However, substances having the C2-C3 double bond and both C3- and C5-OH groups as well as the C4-keto group may show extensive resonance but that this does not necessarily always translate into higher radical stability (Bors and Saran, 1987).

(+)-Catechin failed to indicate a bathochromic shift in the presence of Fe(II), which is similar to the finding by Moridani et al. (2002). However, Mira et al. (2002) found (+)-catechin capable of chelating Cu(II) and a study by Fernandez et al (2002) showed that (+)-catechin chelated Cu(I) as well as Fe(III). In order to effect this metal complexation, the flavanol (+)-catechin which does have the C3-OH group but lacks C4-carbonyl group, must involve the ortho-catechol moiety in

the B ring as metal ion binding site. The present study furthermore demonstrates quercetin, chrysoeriol, luteolin and (+)-catechin to be efficient reducing agents, but with only luteolin and quercetin displaying metal ion chelation properties in the assay used.

From the antioxidant data collected on the rooibos flavonoids, several structure-activity or structure-function relationships were confirmed, viz. (i) the importance of a B ring catechol moiety in the scavenging of free radicals and thereby inhibiting free radical formation as well as the propagation of free-radical reactions by chelating of metal ions; (ii) that scavenging behaviour and phase distribution depends on the degree of hydroxylation of the molecules especially in the B and C rings; (iii) the position of the sugar moiety at either C6 or C8 affects antioxidant efficacy; (iv) that methylation of a B ring hydroxyl group increases the inhibition efficiency; (v) that the C3-OH group is a more important functional group for inhibitory performance than a C4-keto group and (vi) that the introduction of sugar moieties on the C3-OH in ring C of flavonols reduces their antioxidant behaviour due to steric hindrance interactions.

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CHAPTER 5

ANTIMUTAGENIC ACTIVITY OF THE MAJOR FLAVONOIDS OF ROOIBOS (*Aspalathus linearis*)

ABSTRACT

The antimutagenic properties of thirteen of the most prevalent rooibos (*Aspalathus linearis*) flavonoids were compared in the *Salmonella typhimurium* mutagenicity assay using tester strains TA98 and TA100 with 2-acetamido-fluorene (2-AAF) and aflatoxin B_1 (AFB_1) as mutagens, respectively. The mutagenic properties of the flavonoids were also investigated in the presence and absence of metabolic activation using Aroclor-induced rat liver homogenate fraction (S-9). The polyphenols included the dihydrochalcones, aspalathin and nothofagin with their respective flavone analogues, i.e. orientin and isoorientin, and vitexin and isovitexin as well as seven other flavonoids, i.e. luteolin, chrysoeriol, (+)-catechin, quercetin, quercetin-3-*O*-glucoside, hyperoside and rutin. Aspalathin and nothofagin displayed moderate antimutagenic properties. The most effective antimutagen against both mutagens was luteolin that exhibited properties comparable to that of the benchmark epigallocatechin gallate (EGCG). Chrysoeriol was also very effective against AFB_1. Luteolin however showed to be comutagenic with 2-AAF at a low concentration. The antimutagenic properties were associated with specific structural requirements, i.e. the formation of the C ring, the degree of hydrophilicity due to the extent of hydroxylation and *O*-methylation, as well as glycosylation on the A and B rings, the C4-keto group and C2-C3 double bond. Dose response effects were mutagen specific and ranged from typical to atypical to include biphasic as well

as threshold effects. Depending on the flavonoid and the concentration tested antimutagenic (e.g. luteolin and both mutagens tested), comutagenic (e.g. chrysoeriol with 2-AAF), promutagenic (e.g. quercetin-3-*O*-glucoside and 2-AAF) or mutagenic (e.g. quercetin) responses were noticed. Quercetin was the only mutagenic flavonoid. The potent antimutagenic response of rooibos could not be exclusively attributed to any of the monomeric flavonoid constituents tested.

INTRODUCTION

The health benefits of flavonoids are well known and are displayed as a remarkable range of biochemical and pharmacological properties that may significantly affect the function of various mammalian cells (Middelton et al., 2000). The anti-inflammatory, antioxidant, antithrombotic and anticarcinogenic effects are some of the properties that have been under consideration for therapeutical purposes for several human diseases. In this regard, the beneficial properties of Japanese tea are well documented (Yang et al., 2001) and green tea is regarded as one of the major sources of natural flavonoids (Chen and Yen, 1997). The popularity of decaffeinated teas or herbal beverages has also increased as there is growing evidence about the harmful effects of caffeine (Stavric, 1996). A traditional and indigenous South African herbal drink, prepared from rooibos (*Aspalathus linearis*), is subsequently gaining popularity as a health drink (Erickson, 2003) with a relatively low tannin content and no caffeine (Blommaert and Steenkamp, 1978).

Rooibos has a unique flavonoid profile which is enzymatically and chemically altered during processing by oxidation or “fermentation” (Joubert, 1996). The most abundant flavonoids are aspalathin and nothofagin, two C-C linked ß-hydroxy-dihydrochalcone glucosides, which can constitute up to 10% and 1% respectively of the dry unfermented (‘green’) rooibos (Schulz et al., 2003). Aspalathin is unique to rooibos (Koeppen and Roux, 1965) whereas nothofagin is found only in rooibos and *Nothofagus fusca* (Joubert, 1996). It is not known whether the dihydrochalcones are directly linked to the biosynthesis of flavones though chalcones are known to be intermediates in the biosynthesis of flavonoids (Harborne and Baxter, 1999). However, it is possible that the dihydrochalcones can be enzymatically oxidized to the corresponding flavones during “fermentation”. Aspalathin is considered to be the precursor to the flavanones dihydro-orientin and dihydro-isoorientin (Koeppen and Roux, 1966). Marais et al. (2000) demonstrated that oxidative cyclisation of aspalathin resulted in the formation of the flavanones (*S*)- and (*R*)-Eriodictyol-6-*C*-*β*-D-glucopyranoside. Further oxidation could result in the formation of the corresponding flavones orientin and isoorientin. Similarly it is plausible that nothofagin, the B ring monohydroxy analogue of aspalathin, could give rise to vitexin and isovitexin. Other monomeric rooibos flavonoids include rutin, isoquercitrin (quercetin-3-*O*-*ß*-D-glucoside) (Koeppen et al., 1962), luteolin and quercetin (Snyckers and Salemi, 1974), chrysoeriol, vitexin and isovitexin (Rabe et al., 1994) as well as hyperoside (quercetin-3-*O*-β-D-galactoside) (Bramati et al., 2003). (+)-Catechin was reported to occur in very low concentrations in fermented rooibos (Ferreira et al., 1995).

The relative quantities of these compounds differ between the aqueous extracts of fermented and unfermented rooibos (Bramati et al., 2002, 2003; Joubert et al., 2005) as well as between batches of different plantations (Van der Merwe et al., 2006).

The antioxidant properties of rooibos and its major polyphenolic constituents have been established (Yoshikawa et al., 1990; Von Gadow et al., 1997; Joubert et al., 2004), while the antimutagenic properties of fermented and unfermented rooibos as well as its antigenotoxic properties have been investigated (Shimoi et al., 1996; Marnewick et al., 2000; Standley et al., 2001; Edenharder et al., 2002; Van der Merwe et al., 2006).

Information on the biological activities, in particular the antimutagenic activity, of the individual flavonoid constituents is, however, limited. Steele et al. (1985) studied the effect of (+)-catechin against the mutagenicity of various aromatic amines, while the modulating effects of rutin, quercetin (Francis et al., 1989), (+)-catechin (Francis et al., 1989; Choi et al., 1994) and luteolin (Choi et al., 1994) against aflatoxin B_1 (AFB_1)-induced mutagenesis have been reported. The protective effect of rutin, luteolin and quercetin against benzo[a]pyrene (Das et al., 1994), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Edenharder et al., 1993, 1997) and the inhibition of the mutagenicity of three kinds of cyclic nitroarenes by luteolin, quercetin, rutin, isoquercitrin and catechin (Edenharder and Tang, 1997) has been reported. Shimoi et al. (1996) associated the anticlastogenic properties of a rooibos infusion with luteolin. Little is known about the antimutagenic properties of the dihydrochalcones, though MacGregor

and Jurd (1978) found the dihydrochalcones hesperetin and neohesperidin to be non-mutagenic against TA98.

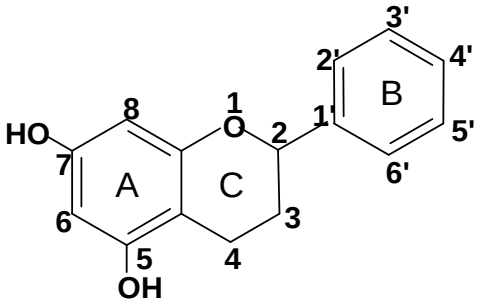
The present study describes the antimutagenicity of the major monomeric flavonoids of rooibos. Epigallocatechin gallate (EGCG), the most abundant (Kuroda and Hara, 1999; Frei and Higdon, 2003) and one of the major antimutagenic principles of Japanese green tea (*Camellia sinensis*) (Kada et al., 1985), was included as reference. The antimutagenic properties of aspalathin and nothofagin and their flavonoid analogues, with emphasis on dose response effects and structural requirements, are reported for the first time.

MATERIALS AND METHODS

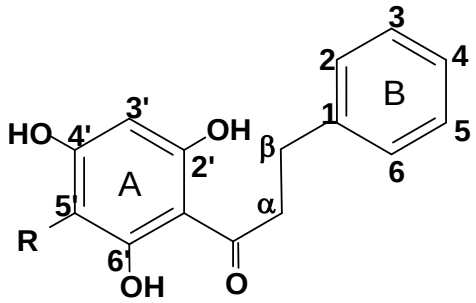
Reagents

Aspalathin and nothofagin were isolated from unfermented rooibos to a purity >95% based on HPLC, NMR and LC-MS at the PROMEC Unit (see Chapter 3). HPLC grade flavonoids, i.e. chrysoeriol, orientin, isoorientin (as homoorientin), vitexin, isovitexin, hyperoside and quercetin-3-*O*-glucopyranoside (isoquercitrin) were obtained from Extrasynthese (Genay, France). Luteolin, quercetin (as quercetin dihydrate), rutin (>95%), (+)-catechin (>98%) and epigallocatechin gallate (>95%, EGCG) were purchased from Sigma-Aldrich S.A. The chemical structures of the various dihydrochalcones and flavonoids utilised in this study are shown in Table 5.1. The mutagens 2-AAF, AFB_1, nicotine adenine dinucleotide phosphate (NADP) and glucose-6-phosphate were acquired from Sigma-Aldrich S.A., bacto agar from Difco Laboratories (Detroit, USA) and

Table 5.1. Chemical structures of the major rooibos flavonoids.



Flavonoid



Dihydrochalcone

	C2=C3 double bond	C3 moiety	C4 C=O	C6	C8	B ring substitution
Dihydrochalcone						
Aspalathin						3, 4-dihydroxy
Nothofagin						4-hydroxy
Flavone						
Luteolin	+	-	+	-	-	3´, 4´-dihydroxy
Chrysoeriol	+	-	+	-	-	4´-hydroxy-3´-methoxy
Orientin	+	-	+	-	CG	3´, 4´-dihydroxy
Isoorientin	+	-	+	CG	-	3´, 4´-dihydroxy
Vitexin	+	-	+	-	CG	4´-hydroxy
Isovitexin	+	-	+	CG	-	4´-hydroxy
Flavanol						
Catechin	-	OH	-	-	-	3´, 4´-dihydroxy
Epigallocatechin gallate[#]	-	Gallate	-	-	-	3´,4´,5´-trihydroxy
Flavonol						
Quercetin	+	OH	+	-	-	3´, 4´-dihydroxy
Isoquercitrin	+	OG	+	-	-	3´, 4´-dihydroxy
Hyperoside	+	OGa	+	-	-	3´, 4´-dihydroxy
Rutin	+	OR	+	-	-	3´, 4´-dihydroxy

OR : *O*-rutinosyl; OG : *O*-glucopyranosyl; R : CG : C-glucopyranosyl; OGa : *O*-galactosyl.
[#]Green tea flavonoid used as reference.

nutrient broth from Oxoid (Hampshire, UK). D-Biotin and L(-)-Histidin were purchased from ICN Biomedicals Inc, (Ohio, USA) and Merck Chemicals Pty. Ltd. (Darmstadt, Germany), respectively. All chemicals (analytical grade) were used as purchased. *Salmonella typhimurium* TA98 and TA100 were obtained from Prof B N Ames, Berkeley University, CA, USA.

Mutagenicity assay

The plate incorporation mutagenicity test was conducted as described by Maron and Ames (1983) and Mortelmans and Zeiger (2000) using tester strains *Salmonella typhimurium* TA98 and TA100 with 2-acetamido-fluorene (2-AAF; 5 µg/plate) and aflatoxin B_1 (AFB_1; 20 ng/plate) as mutagens, respectively. Metabolic activation was achieved by an Aroclor 1254 induced S9 homogenate (0.7 nmole cytochrome P450/mg protein), prepared from male Fischer rats (Maron and Ames, 1983), and incorporated in the S9 mixture at a level of 2 mg protein/ml. Stock solutions of the flavonoids and the mutagens were prepared in DMSO prior to conducting the mutagenic assay. The Ames assay consisted of the addition of overnight bacterial culture (100 µl) to the diagnostic mutagen (100 µl), the flavonoid (100 µl, ranging varying from 0.001-1.2 mM per plate) and 500 µl of S9 mix to 2 ml of top agar at 45°C. The mixture was vortexed, poured onto a minimal glucose plate and incubated at 37°C for 48 h in the dark. Positive and negative (spontaneous) controls in the presence and absence of the diagnostic mutagens respectively, as well as the presence of DMSO and the S9 mixture were included. When the mutagenic response of the diagnostic mutagen was enhanced, the effect of the flavonoid as potential mutagen or promutagen was evaluated in the absence of the diagnostic mutagen with and

without metabolic activation. The percentage inhibition/stimulation of the mutagen-induced response by the flavonoid was calculated using the formula $\{100-\left(\frac{(R_s-R_0)}{(R_p-R_0)}\right)x100\}$, where R_s is the average number of His$^+$ revertants induced in the presence of the flavonoid, R_0 the average number of His$^+$ revertants in the absence of the mutagen (spontaneous revertants) and R_p is the average number of His$^+$ revertants induced by the mutagen. Five repetitions for each concentration were included.

Statistical analyses

All individual groups were independent and tested for normality using the Kolmogorov-Smirnof Test. Levene's Test was used to determine whether the groups had equal variances. Significant group differences were determined by the F-test (equality of variances) or the Welch test (inequality of variances), while the post-hoc Tukey test was used to determine which groups differed significantly. The Student's Two-sample T-test was used to test for group differences when there were only two groups, with the Pooled method for groups with equal variances or the Satterthwaite method for groups with unequal variances. Significant group differences were indicated by $P<0.05$.

RESULTS

The response of the flavonoids in the mutagenicity assay manifested in four ways: as (a) an antimutagen displaying a protective effect due to a decrease in the number of revertants of the positive control; (b) a comutagen enhancing the mutagenic response irrespective of being mutagenic or not; (c) a promutagen

requiring metabolically activation and (d) a direct mutagen not requiring metabolic activation.

A noticeable higher protection was offered by the different rooibos tea flavonoids against AFB_1-induced mutagenesis compared to 2-AAF (Tables 5.2 and 5.3). Aspalathin displayed a moderate antimutagenic effect against 2-AAF and AFB_1 with no clear dose response effect. Orientin and isoorientin displayed a typical protective dose response effect only against AFB_1. At the highest dose, orientin and isoorientin were more effective ($P<0.05$) inhibitors of AFB_1-induced mutagenesis than aspalathin. Orientin exhibited a similar protective effect than aspalathin ($P<0.05$) against 2-AAF, while isoorientin exhibited a significantly higher protective effect at all the concentrations tested. Nothofagin displayed a higher ($P<0.05$) protective effect than aspalathin at the higher concentrations (0.4 and 0.8 mM) against AFB_1 while the opposite was true for 2-AAF. Vitexin exhibited a protective effect similar to that of nothofagin against 2-AAF at 0.4 mM ($P<0.05$) while it offered no protection at 0.08 and 0.8 mM. Isovitexin enhanced ($P<0.05$) the mutagenicity of 2-AAF at the higher concentrations (>0.4 mM) in a typical dose response. Vitexin and isovitexin showed a protective effect similar to that of nothofagin at concentrations ≥0.4 mM against AFB_1, while vitexin exhibited a weaker response than isovitexin ($P<0.05$) at 0.8 mM.

Luteolin was the most effective rooibos antimutagen and exhibited a similar inhibition than EGCG, the green tea flavanol, at 0.8 mM against 2-AAF and AFB_1. However, at lower concentrations (<0.4 mM) luteolin was significantly

Table 5.2. Percentage inhibition (-) or stimulation (+) of the mutagenic response of 2-AAF by various flavonoids against tester strain *Salmonella typhimurium* TA98 in the presence of metabolic activation.

Flavonoid (mM)	% Inhibition (-)/stimulation (+) of mutagenic response										
	1.2	**0.8**	**0.6**	**0.4**	**0.12**	**0.08**	**0.06**	**0.04**	**0.01**	**0.008**	**0.006**
Dihydrochalcones											
Aspalathin	-	(-)27±3bcd[1]	-	(-)30±5abc[1]	-	(-)31±4cde[1]	-	-	-	-	-
Nothofagin	-	(-)19±3bcde[1]	-	(-)19±7cde[1]	-	(-)21±1ef[1]	-	-	-	-	-
Flavone											
Luteolin	(-)100±1[1]	(-)97±2a[1,2]	(-)100±1[1]	(-)96±3h[2]	(-)80±3[3,4]	(-)76±3a[3]	(-)84±5[4]	(-)50±6[5]	(-)7±4[6]	(+)127±53[7]	-
Chrysoeriol	(+)82±9[1]	(+)63±8[2]	(+)45±9[3]	(-)7±6efgh[4]	(-)88±5[5]	(-)57±4b[6]	(-)61±7[6]	(-)19±15[4]	(+)32±8[3]	(+)130±8[7]	-
Orientin	-	(-)35±5bc[1]	-	(-)37±4a[1]	-	(-)32±3cde[1]	-	-	-	-	-
Isoorientin	-	(-)41±8b[1]	-	(-)38±3ab[1]	-	(-)37±1cd[1]	-	-	-	-	-
Vitexin	-	(-)3±10efg[1,2]	-	(-)15±4def[1]	-	(+)3±5h[1,2]	-	-	-	-	-
Isovitexin	-	(+)17±10hi[1]	-	(+)9±5i[1]	-	(-)3±15gh[1]	-	-	-	-	-
Flavanol											
Catechin	(+)20±4[2,3,4]	(-)15±7def[3,4]	(-)22±4[2,3,4]	(-)25±4cd[1,2,3]	(-)33±3[1]	(-)27±2de[1,2]	(-)13±5[4]	-	(-)14±5[5]	-	-
Epigallocatechin gallate[#]	-	(-)95±2a[1]	-	(-)80±3j[2]	-	(-)44±10bc[3]	-	(-)39±6[3,4]	-	(-)27±5[4]	-
Flavonol											
Quercetin	(+)397±11[1]	-	(+)344±23[2]	-	(+)282±22[3]	-	(+)230±9[4]	-	(+)158±5[5]	-	(+)133±10[6]
Quercetin-3-*O*-glucoside	-	(+)222±15[1]	-	(+)153±10[2]	-	(+)142±4i[2,3]	-	(+)121±8[3]	-	(+)90±8[4]	-
Hyperoside	-	(+)14±5gh[1]	-	(-)4±4fgh[2]	-	(-)18±2efg[3,4]	-	(-)14±4[3,4]	-	(-)22±7[4]	-
Rutin	-	(+)14±4ghi[1]	(-)0±4[2]	(-)8±2efg[3]	-	(-)11±4fgh[4]	(-)17±6[5]	-	-	-	(-)47±7[6]

Values are the means±SD of 5 plates per treatment. Values in columns followed by the same letter do not differ significantly (P>0.05). Values in rows followed by the same superscript number do not differ significantly (P>0.05). Average spontaneous revertants (n=40) are 35±6; 2-AAF positive control 397±33; spontaneous revertants subtracted from response to calculate % inhibition. Concentration of 2-AAF: 5µg/plate. Concentration of compound indicated as mM in final volume. [#]Used as reference.

Table 5.3. Percentage inhibition (-) or stimulation (+) of mutagenic response of AFB_1 by various flavonoids against tester strain *Salmonella typhimurium* TA100 in the presence of metabolic activation.

Flavonoid (mM)	% Inhibition (-)/Stimulation (+) of mutagenic response										
	1.2	**0.8**	**0.6**	**0.4**	**0.12**	**0.08**	**0.06**	**0.04**	**0.01**	**0.008**	**0.006**
Dihydrochalcones											
Aspalathin	-	(-)43±7gf[1]	-	(-)48±5bcdef[1]	-	(-)33±3bcde[2]	-	-	-	-	-
Nothofagin	-	(-)59±3cde[1]	-	(-)59±4b[1]	-	(-)33±8bcdef[2]	-	-	-	-	-
Flavone											
Luteolin	(-)112±2[1]	(-)96±2a[2,3]	(-)100±9[1,2]	(-)91±5a[2,3]	(-)85±6[3,4]	(-)89±4a[2,3]	(-)86±6[3,4]	(-)87±2[3,4]	(-)75±8[4,5]	(-)62±5[5]	-
Chrysoeriol	(-)82±5[1,2]	(-)73±5b[2]	(-)74±4[2]	(-)82±3a[1,2]	(-)82±5[1,2]	(-)83±2a[1]	(-)76±2[1,2]	-	(-)54±4[3]	-	-
Orientin	-	(-)59±3cde[1]	-	(-)46±8bcdefgh[2]	-	(-)35±3bcd[3]	-	-	-	-	-
Isoorientin	-	(-)66±10bc[1]	-	(-)50±7bcde[2]	-	(-)23±5cdefgh[3]	-	-	-	-	-
Vitexin	-	(-)51±4defg[1]	-	(-)51±4bcd[1]	-	(-)15±5ghi[2]	-	-	-	-	-
Isovitexin	-	(-)62±5bcd[1]	-	(-)50±3bcdef[2]	-	(-)37±4bc[3]	-	-	-	-	-
Flavanol											
Catechin	(-)72±11[1]	(-)40±8g[2,3]	(-)45±9[2]	(-)26±14i[3,4]	(-)35±5[2,3]	(-)8±4hij[4]	(-)29±1[3]	-	(-)42±2[5]	-	-
Epigallocatechin gallate[#]	-	(-)101±4a[1]	-	(-)93±3a[1]	-	(-)69±7k[2]	-	(-)50±4[3]	-	(+)2±7[4]	-
Flavonol											
Quercetin	⊗	-	⊗	-	(+)100±12[1]	-	(+)32±7[2]	-	(-)9±3[3]	-	(-)19±5[3]
Quercetin-3-*O*-glucoside	-	(+)26±8h[1]	-	(-)18±3i[2]	-	(-)35±7bc[3]	-	(-)29±5[3]	-	(-)31±5[3]	-
Hyperoside	-	(-)55±2cdef[1]	-	(-)51±7bc[1]	-	(-)27±5bcdefg[2,3]	-	(-)20±8[3]	-	(-)36±6[2]	
Rutin	-	(-)47±3efg[1]	(-)46±7[1]	(-)48±9bcdefgh[1,2,3]	-	(-)14±4ghij[2,3,4]	(-)27±9[3]	(-)44±12[1,2,3]	-	-	(-)36±10[1,2]

Values are the means±SD of 5 plates per treatment. Values in columns followed by the same letter do not differ significantly (P>0.05). Values in rows followed by the same superscript number do not differ significantly (P>0.05). Average spontaneous revertants (n=40) are 141±15; AFB_1 positive control 416±38; spontaneous revertants subtracted. Concentration of AFB_1: 20ng/plate. Concentration of compound indicated as mM in final volume. ⊗Cytotoxic effect. [#]Used as reference.

more effective than EGCG against both 2-AAF and AFB_1, except that a comutagenic effect was noticed against 2-AAF at 0.008 mM. Chrysoeriol exhibited a comutagenic effect at the highest and lower concentrations against 2-AAF (biphasic response) while it protected at concentrations 0.4-0.04 mM. Against AFB_1 it protected at all the concentrations tested and exhibited similar antimutagenic properties than luteolin at 0.4, 0.12 and 0.08 mM. However, it offered less protection at concentrations ≤0.06 mM and ≥0.6 mM. Chrysoeriol exhibited a higher protective effect than EGCG at concentrations ≤ 0.08 mM.

(+)-Catechin displayed a similar protection than aspalathin and nothofagin against 2-AAF at all the concentrations tested. It exhibited a higher protective effect than isovitexin against 2-AAF, similar to vitexin at ≥0.4 mM and a lower protective effect when compared to orientin and isoorientin. Against AFB_1, (+)-catechin displayed a biphasic response with a high protection at high and low concentrations. (+)-Catechin showed a similar protection than aspalathin against AFB_1 at 0.8 mM, but a lower effect than nothofagin and their structural flavone analogues at all the concentrations tested.

Rutin reduced the number of 2-AAF-induced revertants in a typical dose response. Rutin and hyperoside exhibited similar antimutagenic responses against 2-AAF at 0.4 and 0.08 mM, but stimulated mutagenic response ($P<0.05$) at the highest concentration (0.8 mM). They exhibited a weaker protective response than aspalathin, orientin, isoorientin and nothofagin at ≤0.4 mM. Both rutin and hyperoside exhibited a similar protective effect than aspalathin, nothofagin and their structural analogues against AFB_1, except at

0.08 mM where rutin had a protection similar to vitexin. Both rutin and hyperoside exhibited a biphasic antimutagenic response, similar to that of (+)-catechin and luteolin, against AFB_1.

Quercetin enhanced the mutagenic response of 2-AAF at all the concentrations in a typical dose dependent manner. It was however cytotoxic against TA100 at concentrations higher than 0.12 mM. Quercetin stimulated AFB_1-induced mutagenesis at concentrations ≥0.06 mM while a protective effect was noticed at a concentration <0.01 mM. Quercetin-3-*O*-glucoside increased the mutagenic response of 2-AAF in a dose dependent manner although to a lesser extent than quercetin. It lacked any toxic effect against TA100 while inhibiting AFB_1-induced mutagenesis except at the highest level (0.8 mM) tested where the mutagenicity was enhanced.

The flavonoids that enhanced the mutagenic response of 2-AAF and AFB_1 were tested as possible mutagens in the absence and presence of the S9 mixture for metabolic activation (Tables 5.4 and 5.5, respectively). Responses are indicated as the number of revertants including the number of spontaneous revertants. Quercetin and quercetin-3-*O*-glucoside were mutagenic towards both strains in the absence of metabolic activation while evidence of a promutagenic response ($P<0.05$) was noticed in the presence of metabolic activation. Considering the additive effect of 2-AAF in the absence and presence of quercetin, an antimutagenic and comutagenic effect was observed at higher (>0.06mM) and lower (0.01mM) concentrations, respectively. Quercetin-3-*O*-glucoside exhibited a similar, though less marked, response. When considering the interaction with AFB_1, an antimutagenic effect was

Table 5.4. Evaluation of the mutagenic activity of flavonoids found to be comutagenic towards 2-AAF using *Salmonella typhimurium* TA98.

Flavonoid (mM)			Number of revertants											
	2-AAF	S9	1.2	0.8	0.6	0.4	0.12	0.08	0.06	0.04	0.01	0.008	0.006	0.001
Quercetin	+	+	1720±39a[1]	-	1542±77a[2]	-	1332±74a[3]	-	1156±30a[4]	-	910±48a[5]	-	828±32a[6]	796±22a[6]
	-	+	1448±28b[1]	-	1380±41b[1]	-	1234±36a[2]	-	1088±35b[3]	-	317±32b[4]	-	235±12b[5]	51±5b[6]
	-	-	1310±26c[1]	-	1324±22b[1]	-	1126±48b[2]	-	672±59c[3]	-	192±42c[4]	-	96±9c[5]	40±9b[5]
Quercetin-3-*O*-glucoside	+	+	-	1172±53a[1]	-	921±37a[2]	-	890±15a[2,3]	-	823±27[3]	-	725±27[4]	-	-
	-	+	-	1164±34a[1]	-	697±37b[2]	-	237±39b[3]	-	-	-	-	-	-
	-	-	-	752±54b[1]	-	367±42c[2]	-	109±7c[3]	-	-	-	-	-	-
Hyperoside	+	+	-	415±17[1]	-	355±12[2]	-	295±13[3,4]	-	335±27[3,4]	-	295±22[4]	-	-
	-	+	-	33±10	-	26±2	-	24±6	-	-	-	-	-	-
	-	-	-	21±3	-	20±4	-	17±2	-	-	-	-	-	-
Rutin	+	+	-	388±14[1]	368±14[2]	310±29[3]	-	308±18[4]	305±22[5]	-	-	-	197±27[6]	-
	-	+	-	-	38±2	-	-	-	38±4	-	-	-	35±4	-
	-	-	-	-	37±3	-	-	-	36±5	-	-	-	-	-
Isovitexin	+	+	-	432±37[1]	-	410±22[1]	-	400±8[1]	-	-	-	-	-	-
	-	+	-	34±7	-	34±4	-	37±3	-	-	-	-	-	-
	-	-	-	17±4	-	18±4	-	22±3	-	-	-	-	-	-
Chrysoeriol	+	+	623±9[1]	551±28[2]	437±21[3]	343±19[4]	123±6[5]	172±14[6]	123±26[6]	347±5[4]	485±58[3]	808±26[7]	-	-
	-	+	37±1	-	40±5	-	35±5	-	37±3	-	39±4	-	-	-
	-	-	35±2	-	43±1	-	37±5	-	35±5	-	40±2	-	-	-

Values are the means ± SD of 5 plates per treatment. Values in columns followed by the same letter do not differ significantly (P>0.05). Values in rows followed by the same superscript number do not differ significantly (P>0.05). Average spontaneous revertants (n=40) are 35±6; 2-AAF positive control 397±33; spontaneous revertants not subtracted from response. Concentration of 2-AAF: 5µg/plate. Concentration of compound indicated as mM in final volume.

Table 5.5. Evaluation of the mutagenic activity of the flavonoids found to be comutagenic towards AFB_1 using *Salmonella typhimurium* TA100.

Flavonoid (mM)			Number of revertants											
	AFB_1	S9	1.2	0.8	0.6	0.4	0.12	0.08	0.06	0.04	0.01	0.008	0.006	0.001
Quercetin	+	+	⊗	-	⊗	-	628±31a[1]	-	494±18a[2]	-	350±19a[3]	-	332±20a[3]	321±23a[3]
	-	+	⊗	-	⊗	-	577±20b[1]	-	454±61a[2]	-	289±22b[3]	-	230±11b[3]	149±10b[4]
	-	-	431±30[1]	-	401±13[1]	-	317±6c[2]	-	242±19b[3]	-	161±18c[4]	-	157±8c[4]	143±15b[4]
Quercetin-3-*O*-glucoside	+	+	-	519±22a[1]	-	422±10a[2]	-	346±19a[3]	-	362±14[3]	-	357±11[3]	-	-
	-	+	-	486±40a[1]	-	376±16b[2]	-	239±14b[3]	-	-	-	-	-	-
	-	-	-	279±15b[1]	-	252±10c[1]	-	202±16c[2]	-	-	-	-	-	-

Values are the means±SD of 5 plates per treatment. Values in columns followed by the same letter do not differ significantly (P>0.05); if letters differ, then P<0.05. Values in rows followed by the same superscript number do not differ significantly (P>0.05); if numbers differ, then P<0.05. Spontaneous revertants not subtracted. Average spontaneous revertants (n=40) are 141±15; AFB_1 positive control 416±38. Concentration of AFB_1: 20ng/plate. Concentration of compound indicated as mM in final volume. ⊗Cytotoxic effect.

noticed for both these flavonoids at ≤0.01 mM for quercetin and ≤0.08 mM for quercetin-3-*O*-glucoside. Rutin, hyperoside, isovitexin and chrysoeriol were found to be non-mutagenic against TA98 with/without the mutagen and with/without metabolic activation.

DISCUSSION

Varying effects of the flavonoids on the mutagenic behaviour of 2-AAF and AFB_1 are noticed depending on the mutagen and the concentration of the specific flavonoid. Dose response effects are complex and specific responses of individual flavonoids against the mutagens include: (i) a typical dose response where the inhibitory and/or enhancing effect was directly related to the concentration of the flavonoid used, e.g. EGCG and quercetin; (ii) a no dose response effect, e.g. aspalathin and nothofagin against 2-AAF where a threshold was observed at the three concentrations tested; (iii) a non-typical dose response effect reflecting a biphasic shape, e.g. chrysoeriol yielded a comutagenic effect against 2-AAF at high and low concentrations with an antimutagenic effect at intermediate concentrations. Rutin showed strong protection against AFB_1 at high and low concentrations while weaker protection was noticed at intermediate concentrations and (iv) combinations of the aforementioned responses, for example (+)-catechin and rutin against 2-AAF as well as quercetin-3-*O*-glucoside against AFB_1 that displayed a comutagenic effect at the highest concentration but an antimutagenic effect at lower concentrations. Luteolin also displayed a dose dependent behaviour against 2-AAF when the potent antimutagenic response changed into a comutagenic response at the lowest concentration evaluated.

The higher protection exhibited by the different rooibos tea flavonoids against AFB_1- than 2-AAF-induced mutagenesis could be attributed to differences in the metabolic pathways yielding the active mutagenic intermediate. Several electrophilic products are derived from 2-AAF during a two-step activation process that involves both the microsomal and cytosolic enzymes present in the S9-mixture to yield *N*-hydroxy-2-AAF and the acetylated *N*-acetoxy-2-AAF (Heflich and Neft, 1994). In contrast, cytochrome P450 forms the single electrophilic AFB_1-8,9-epoxide from AFB_1 (Bailey and Williams, 1993; Catterall et al., 2003). A study by Yen and Chen (1995) on the antimutagenic effect of green, pouchong, oolong and black tea against five different mutagens showed that the highest protection was against AFB_1 using TA98 and TA100. Van der Merwe et al. (2006) also found aqueous extracts of rooibos and honeybush to offer better protection against mutagenesis induced by AFB_1 in TA100 than 2-AAF-induced mutagenesis in TA98.

At present little information is available on the bioavailability and metabolism of the rooibos flavonoids, but biotransformation could either decrease or increase their biological activity (Canivenc-Lavier et al., 1996; Muto et al., 2001). Since the liver homogenate fraction utilised in the *Salmonella* assay contains phase I and phase II drug metabolising enzymes, it is possible that the flavonoids could be metabolically transformed with some of the metabolites, rather than the parent flavonoid, mediating the actual biological response (Breinholt et al., 2002). The primary site of flavonoid biotransformation by rat liver microsomes is the 3′- and 4′-position on the B-ring, resulting in major end-products with 3′,4′-dihydroxylated B-rings (Nielsen et al., 1998; Breinholt et al., 2002). Flavonoids

that lack hydroxyl groups in the B-ring or have a 4'-hydroxy group are hydroxylated by microsomal enzymes to the corresponding catechol moiety (Nielsen et al., 1998). Another metabolic process that may involve the flavonoids is conjugation reactions that include methylation, sulfation and glucuronidation (Manach et al., 2004). In the present study the mutagenic potency of both quercetin and quercetin-3-*O*-glucoside were significantly enhanced by metabolic activation.

The moderate antimutagenic effects exhibited by aspalathin and nothofagin and the lack of a dose response effect suggested an apparent upper threshold against both mutagens. It is also possible that a catechol moiety could be formed on the B ring during metabolism, resulting in the conversion of nothofagin, as well as vitexin and isovitexin, into aspalathin, orientin and isoorientin, respectively. This could imply that the threshold effects noticed could actually be attributed to the metabolites of nothofagin but this hypothesis would require further investigation. The cyclisation of aspalathin to a flavanone with further oxidation to a flavone would influence its antimutagenic potential. Orientin and isoorientin were more effective than their precursor molecule, aspalathin, against AFB_1-induced mutagenesis at the highest concentration tested. However, the protection displayed by nothofagin and its flavone analogues, vitexin and isovitexin, against AFB_1 were comparable while the flavone analogues exhibited less protection than nothofagin against 2-AAF. At higher concentrations isovitexin even stimulated the mutagenic response. These results confirm the suggestion by Edenharder et al. (1993) that the C

ring is of some importance but not a prerequisite for the antimutagenicity of a flavonoid.

The hydrophilic nature of flavonoids is known to be an important determinant of its antimutagenic function (Edenharder and Tang, 1997; Francis et al., 1989). The two major determinants of the hydrophilic nature of flavonoids are the hydroxylation status and the presence and position of a sugar moiety. Huang et al. (1983) has linked an increase in the number of phenolic hydroxyl groups of a flavonoid to an increased antimutagenic activity against benzo[a]-pyrene and TA100, while Edenharder et al. (1993) postulated that the increase in the number of hydroxyl groups and the subsequent increased polarity reduced the antimutagenic potential in the case of TA98 and IQ-induced mutagenesis. The number of hydroxyl groups could therefore either enhance or decrease the protective effect depending on the flavonoid subgroup and the type of mutagen used. Aspalathin, orientin and isoorientin with a catechol moiety as the B ring, were more effective inhibitors of 2-AAF-induced mutagenesis than their C4-monohydroxylated counterparts nothofagin, vitexin and isovitexin. In the case of AFB_1, however, nothofagin was a more efficient antimutagen than aspalathin while the flavone analoques displayed similar protective properties. Amongst the flavanols investigated, (+)-catechin exhibited a far lower protective effect against AFB_1- and 2-AAF-induced mutagenesis when compared to EGCG that has a trihydroxy arrangement on the B ring. Methyl etherification of primarily the 3'-hydroxyl groups, catalyzed by catechol-*O*-methyl transferase (COMT) (Manach et al., 2004) decrease the polarity and had varying effects on mutagenesis. *O*-Methylation of the C3´-OH of luteolin, yielding chrysoeriol,

resulted in a significant to a marked decrease in protection against AFB_1. Against 2-AAF it exhibited a significantly lower protective effect, becoming comutagenic again at higher concentration levels. Methylation of luteolin at the C4´-OH group yields the structural isomer of chrysoeriol viz., diosmetin (3',5,7-trihydroxy-4'-methoxyflavone) which also exhibited a comutagenic response when using IQ as a mutagen against TA98 (Edenharder et al., 1997).

Glycosylation at the A ring decreased the antimutagenic activity as seen when comparing the responses of orientin and isoorientin with their aglycone luteolin. This decrease in protection could be attributed to steric hindrance or an increased polarity resulting in the flavonoid being prevalent in a different cellular compartment than the activated mutagen. The relative position of the sugar moiety at C6 or C8 seems not important as a similar degree of protection was displayed by both isomers against AFB_1 and 2-AAF. However, on comparing vitexin and isovitexin, the C6 and C8 position for the sugar moiety markedly affected protective effects as isovitexin displayed a comutagenic effect against 2-AAF while exhibiting a stronger antimutagenic effect against AFB_1 at a low concentration. The degree of glycosylation of the C3-OH interfered in the mutagenic activity of the flavonol quercetin and the decrease of mutagenic activity was more pronounced against AFB_1. The mono-3-*O*-glucoside of quercetin still exhibited a mutagenic effect but to a lesser extent than the aglycone quercetin. The presence of a 3-*O*-galactose (hyperoside) or a 3-*O*-rutinose (rutin) completely inhibited the mutagenic effect to display an antimutagenic effect. This effect was less pronounced when the mutagen was 2-AAF. Francis et al. (1989) also found that rutin exhibited better protection

against AFB_1 than quercetin. It appeared as if the modulation of mutagenesis depended, apart from the kind of mutagen, on the concentrations of the flavonoid, i.e. at high concentration levels, the antimutagenic activity was masked by a mutagenic response. Quercetin and quercetin-3-*O*-glucoside enhanced the mutagenic response of 2-AAF by acting as either a direct mutagen, promutagen or comutagen. The former two actions were displayed against AFB_1 as well.

The mutagenic properties of flavones and flavonols was suggested to be due to the interaction with DNA as the C2,C3-double bond ensured a planar structure that facilitated intercalation (Webb and Ebeler, 2004). Another prerequisite was a C4'-OH on the B-ring to stabilize the binding between flavonoid and DNA by hydrogen bonding. Weak intercalation was observed when stabilization was due to hydrogen bonding of the conjugated glucose. 2-AAF is an intercalating mutagen (Mortelmans and Zeiger, 2000) when using tester strain TA98 and it was thought that the comutagenic or mutagenic responses of the flavonoids could be related to the observations of Webb and Ebeler (2004). Isovitexin, for instance, met the structural requirements described above and was comutagenic in TA98. However, isovitexin failed to act as promutagen or direct acting mutagen. Orientin was found to moderately intercalate with DNA (Webb and Ebeler, 2004), but acted only as an antimutagen.in the present study. The intercalation of flavonoids into DNA, therefore, does not appear to explain the comutagenic or mutagenic effects of these compounds.

Aspalathin, orientin and isoorientin are known as powerful antioxidants (Joubert et al., 2004) with the catechol moiety for aspalathin and adding to

that the C3-OH-group and C2-C3 double bond as key structural requirements for the corresponding flavones (Rice-Evans, 2001). The structural requirements responsible for antioxidant and antimutagenic activity closely resemble each other although the relative contribution towards the antimutagenic activity is not clear as yet. A study with green tea catechins showed that their *in vitro* antioxidant activity in the $DPPH^{\bullet}$, superoxide radical anion scavenging and hydrogen peroxide scavenging assays correlated with their antimutagenic activity against TA102 using *tert*-butylhydroperoxide or hydrogen peroxide (Geetha et al., 2004). In this regard EGCG is known to be to be an efficient antioxidant (Hu and Kitts, 2001) as well as antimutagen. Edenharder and Grünhage (2003) assayed flavonoids like fisetin, rutin, luteolin, quercetin and isoquercitrin to name a few, with $DPPH^{\bullet}$ and in the *Salmonella* strain TA102 with peroxide mutagens CHP and BHP. Their conclusion also was that the antimutagenic activities of flavonoids against the peroxide mutagens are mainly caused by radical scavenging. Hatch et al. (2000), however, after having statistically compared the activities of 39 diverse flavonoids from a database, concluded that the antioxidant or radical scavenging properties of the flavonoids are probably not involved in the inhibition of mutagenesis.

A similar trend was noticed in the present study where the antimutagenic potency of the major rooibos flavonoids did not correlate with their potent antioxidant properties. Aspalathin was an efficient antioxidant (Joubert et al., 2004), while it was not an efficient antimutagen. Luteolin and chrysoeriol were the most effective antimutagenic rooibos flavonoids with similar inhibitory

effects to that of EGCG. Luteolin also exhibited a far better protective effect than (+)-catechin, which is in agreement with studies conducted by Choi et al. (1994), but luteolin was a poor DPPH$^{\bullet}$ scavenger (Hirano et al., 2001) although it was more effective than EGCG in inhibiting LDL oxidation (Hirano et al., 2001). As the levels of luteolin and chrysoeriol are extremely low in unfermented and fermented rooibos extracts (Bramati et al. 2003, 2002) their contribution towards the antimutagenic properties of rooibos, can not yet be defined. Studies are currently in progress to characterise the antimutagenic principles responsible for the antimutagenic properties of rooibos.

The underlying mechanisms involved in the antimutagenic behaviour are not known at present but could be related to a critical balance between different processes involving the metabolism of the specific flavonoid as well as the stabilisation of and/or interference with metabolic enzymes involved in carcinogen metabolism.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Bioactivity-guided fractionation was used to identify the most potent antioxidant and antimutagenic fractions from unfermented rooibos (*Aspalathus linearis*), as well as the bioactive principles for the most potent antioxidant fractions. The *Salmonella* mutagenicity test using tester strain TA98 and metabolically activated 2-acetoaminofluorene was used to assess the antimutagenic potential of the different extracts and fractions, while the antioxidant potency was assessed by two different *in vitro* assays, i.e. the inhibition of Fe(II) induced microsomal lipid peroxidation and the scavenging of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation.

The methanol extract of unfermented rooibos exhibited higher antimutagenic and antioxidant activity than the chloroform extract. A decrease in protection against 2-AAF induced mutagenesis was observed with the less polar XAD fractions.

Successive fractionation of the two XAD fractions most active in the $ABTS^{\bullet+}$ assay led to both aspalathin and nothofagin being isolated for the first time to a purity of >95%.

Thirteen flavonoids of rooibos were compared in the two antioxidant assays as mentioned *vide infra* as well as a metal chelating assay to compare structural

activities of the dihydrochalcones aspalathin and nothofagin, their flavone analogues orientin and isoorientin (from the precursor aspalathin) and vitexin and isovitexin from nothofagin, the flavone aglycones luteolin and chrysoeriol as well as four flavonols from rooibos, i.e. quercetin, isoquercitrin (quercetin-3-*O*-glucoside), hyperoside (quercetin-3-*O*-galactoside) and rutin (quercetin-3-*O*-rutinoside). The flavanol (+)-catechin was also included while epigallocatechin gallate (EGCG), the major active principle from *Camellia sinensis* was used as benchmark.

Aspalathin, the dihydrochalcone unique to rooibos, was a very efficient inhibitor of lipid peroxidation and scavenger of $ABTS^{\bullet+}$, while nothofagin was equipotent to aspalathin in the $ABTS^{\bullet+}$ assay, but had the lowest inhibitory effect (highest IC_{50} value) of all the flavonoids tested in the lipid peroxidation assay. Neither aspalathin nor nothofagin however responded in the metal chelating assay. Orientin and isoorientin had lower IC_{50} values for both antioxidant assays than vitexin and isovitexin. Both the 3′,4′-dihydroxy flavones responded in the metal chelation assay, while the 3′-monohydroxy flavones did not. The introduction of sugar moieties on the flavonol C3-OH reduced the efficacy of quercetin in both assays, rendering isoquercitrin and hyperoside the weakest inhibitor of the flavonols in the $ABTS^{\bullet+}$ and lipid peroxidation assays respectively. The order of increasing IC_{50} values amongst the monomeric rooibos flavonoid aglycone for both the lipid peroxidation and $ABTS^{\bullet+}$ assays, would be quercetin>(+)catechin>chrysoeriol> luteolin.

The thirteen flavonoids of rooibos named above were also investigated in the *Salmonella typhimurium* mutagenicity assay, again using EGCG as benchmark. Strains TA98 and TA100 utilizing 2-acetamido-fluorene (2-AAF) and aflatoxin B_1 (AFB_1) respectively, were used. Mutagenesis of flavonoids was also investigated in the absence of the diagnostic mutagen, with and without metabolic activation. The dose response effects obtained were concentration dependent and mutagen specific and suggested antimutagenic, comutagenic, promutagenic or mutagenic behaviour.

Neither aspalathin nor nothofagin displayed potent antimutagenic properties against both the mutagens evaluated while luteolin was the most effective antimutagen. The antimutagenic activity of orientin and isoorientin, the oxidation products of aspalathin, was either increased or decreased to that of aspalathin depending on the concentration and the specific mutagen used. The antimutagenic behaviour of rooibos could not be solely attributed to any of these monomeric flavonoid constituents.

To conclude:

1. There appears to be no link between the antioxidant and antimutagenic activities of the major rooibos flavonoids.
2. Aspalathin, the flavonoid unique to rooibos and occurring in large quantities in unfermented rooibos, was shown to be the major contributor to antioxidant activity in quantity and potency. As antioxidant, aspalathin is

equipotent to quercetin and the most active flavonoid of *Camellia sinensis*, epigallocatechin gallate (EGCG). However, aspalathin demonstrated only moderate antimutagenic behaviour against both the mutagens evaluated and it most probably contributes towards the antimutagenic property of rooibos due to its relatively higher concentration.

3. Luteolin displayed the strongest antimutagenic activity of the known rooibos flavonoids while it did not show strong antioxidant properties in $ABTS^{\bullet+}$ scavenging and microsomal lipid peroxidation assays. It also occurs in small quantities in the more polar fractions of unfermented rooibos.

4. Nothofagin displayed radical scavenging properties similar to aspalathin but was the least potent rooibos flavonoid in the antiperoxidative ability. It also displayed moderate antimutagenicity against both the mutagens evaluated.

Both the major dihydrochalcones of rooibos play an important role in the radical scavenging ability of rooibos, while aspalathin is the major contributor in the inhibition of Fe(II)-induced lipid peroxidation. Fermentation with a subsequent decrease in aspalathin and nothofagin levels could impair this very important property of rooibos.

Possible future investigations

1. Fractionation of the first, non-polar fraction of the XAD column to isolate and identity a possible active antimutagenic compound utilizing activity-guided fractionation.
2. An investigation into a possible synergism between aspalathin and its two flavone isomers, i.e. isoorientin and orientin with regards to enhancing antioxidant or antimutagenic activities. Orientin and isoorientin are abundant in unfermented rooibos and occur in quantities similar to aspalathin in fermented rooibos and both can be considered moderate antioxidants and antimutagens.
3. A disadvantage of the Fe(II)-induced lipid peroxidation assay as used in this investigation was that it did not distinguish between the radical scavenging nor metal chelating function of the flavonoid as the mechanism to inhibit lipid peroxidation, i.e. the role of the flavonoid could not be defined as that of radical chain breaker nor as preventor of initiation. The catechol moiety in the B ring as well as the 4-keto-C6′-OH structure renders aspalathin a potential potent metal ion chelator but this property still needs further investigation.

ADDENDA

ADDENDUM 3A

Background to IC_{50} values as calculated by Prism Version 5.00 for Windows; GraphPad Software, adapted from the on-line manual to the software.

All schematic representations of graphs, except the first one that was drawn by myself, are from the GraphPad Software manual.

A dose-response curve represents a concentration-dependent response and is loosely used to describe *in vitro* experiments where you apply a known concentration of drugs.

The IC_{50} is defined as the concentration of inhibitor (antioxidant) that provokes a response halfway between the baseline (bottom) and maximum response (top) (figure 3G.1). **It is impossible to define the IC_{50} without having defined the baseline and maximum response first.** (Note: inhibitory equations run downhill with a negative slope to fit the IC_{50}; stimulation equations run uphill to fit the EC_{50}. There is no fundamental difference in the calculations, but the graphs available were for EC_{50} calculations.)

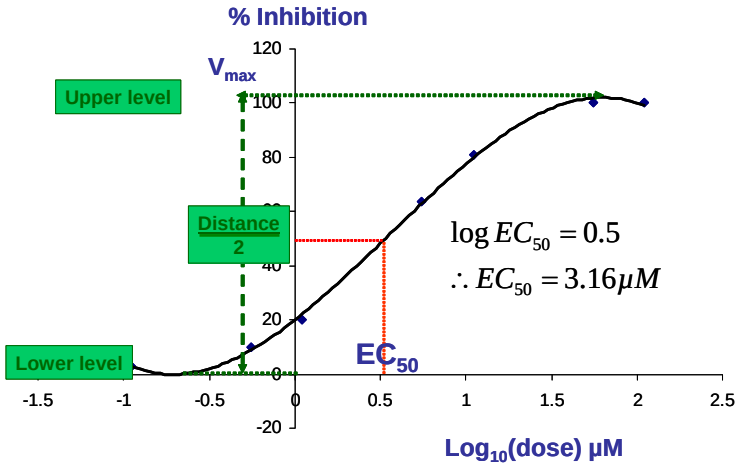


Figure 3G.1 Sigmoidal response curve to show calculation of EC_{50}.

The baseline response need not be 0%; neither the maximum 100% (figure 3G.2).

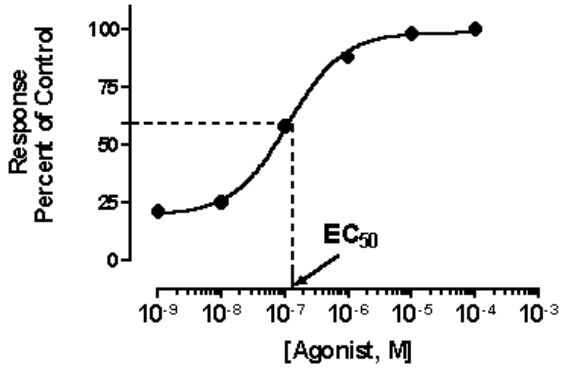


Figure 3G.2 Sigmoidal response curve showing bottom plateau at about 22% percent response of control.

The relationship between dose and response in a system following mass-action kinetics is hyperbolic, not sigmoidal. The dose-response curve becomes sigmoidal only when the response is plotted against the logarithm of the dose.

The model (from the Prism GraphPad Software) used to fit the data of this study, was $y = Bottom + \frac{(Top - Bottom)}{\left[1+10^{((LogEC50-x)*Hillslope)}\right]}$. This dose-response model has four parameters that need to be met: the bottom plateau, the top plateau, the IC_{50} and the slope factor. which was not constrained to a standard value in this case. The main goal of fitting the dose-response curve is to determine the best-fit value of the IC_{50} on all four parameters. If the top and bottom plateaus are not well defined, or if the hill slope is too steep, the IC_{50} will be uncertain with wide lower and upper confidence levels (Manual to Prism 5.00 for Windows; GraphPad Software). These problems can be solved by increasing the number of points to analyze.

If the model fitted the data points, the software calculates the $logIC_{50}$ value and also gives the corresponding IC_{50} value. A standard error value is only shown for $logIC_{50}$ values, and not IC_{50} values, on the results sheet. A typical results sheet is shown below (figure 3G.3).

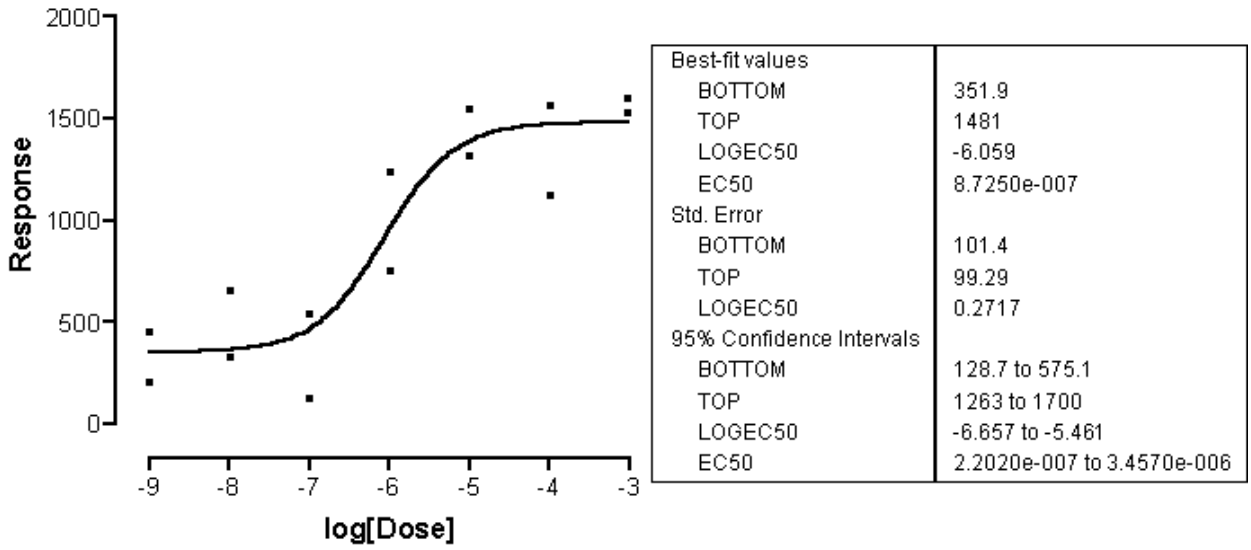


Figure 3G.3 Typical results sheet generated by GraphPad Software.

The standard error is derived from the curve fitting process, and the fitted parameter (X variable) is log EC_{50}, not EC_{50}. Similarly statistical analyses are performed on the logIC_{50} values.

The software was however able to determine IC_{50} values with well defined lower and upper 95% confidence intervals. This confidence interval represents a range of concentrations within which the IC_{50} will be found with 95% certainty. Significant differences shown for log IC_{50} values may be applied to the IC_{50} values (Dr Martin Kidd, Statistical Consultant, University of Stellenbosch; personal communication). When computing IC_{50} values for a range of points, the customary mean value ± standard deviation can therefore not be used. The correct and most reader-friendly way to present these values would be to report the computed IC_{50} value showing the upper and lower levels of the 95% confidence interval with the R^2 value to show the degree of fit of the points to the model.

The hillslope indicates the kinetics of an inhibition reaction. Sigmoidal dose response curve with different hill slopes are shown in Figure 3G.4.

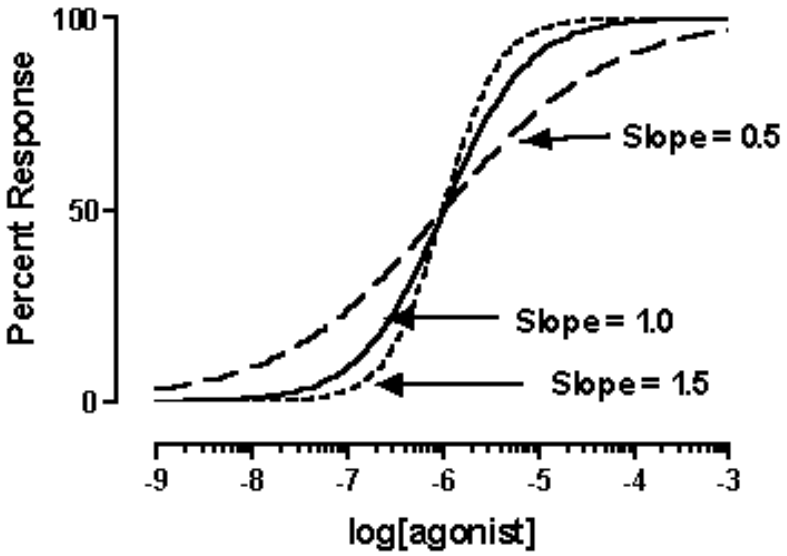
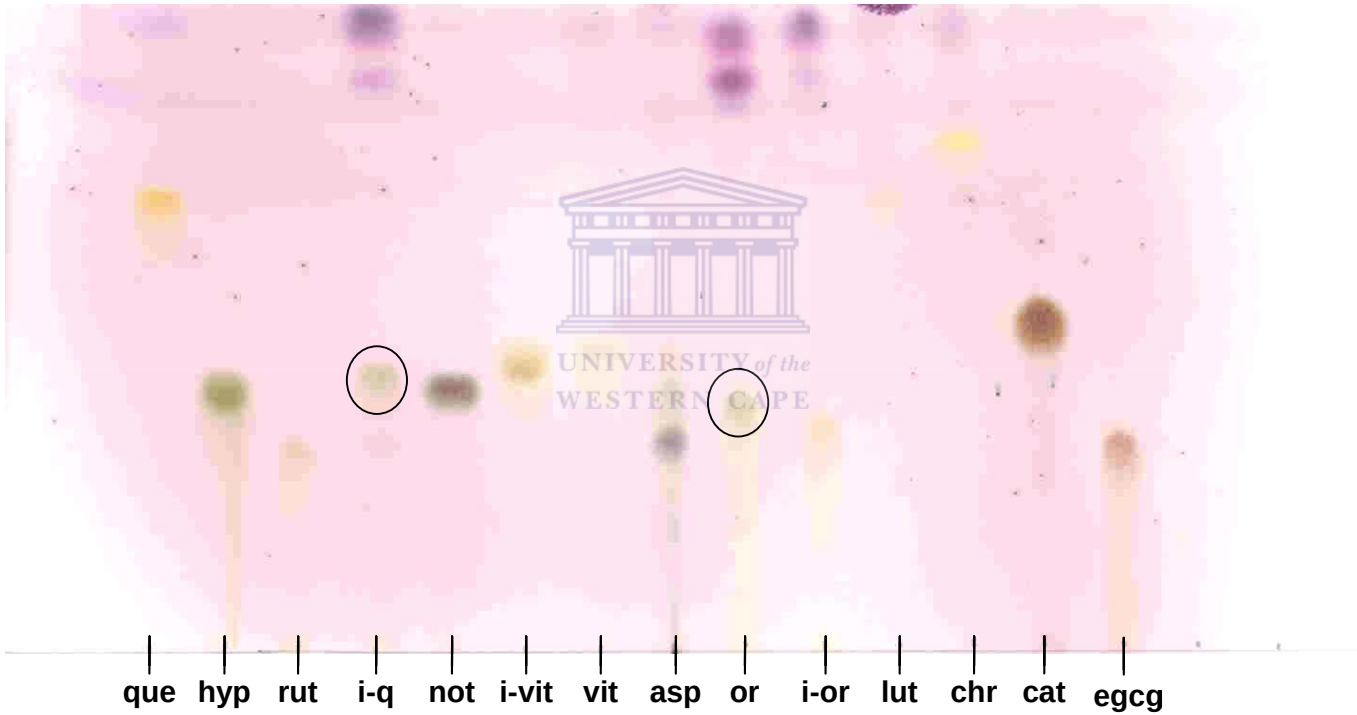


Figure 3G.4 Sigmoidal dose response curves with different hill slopes.

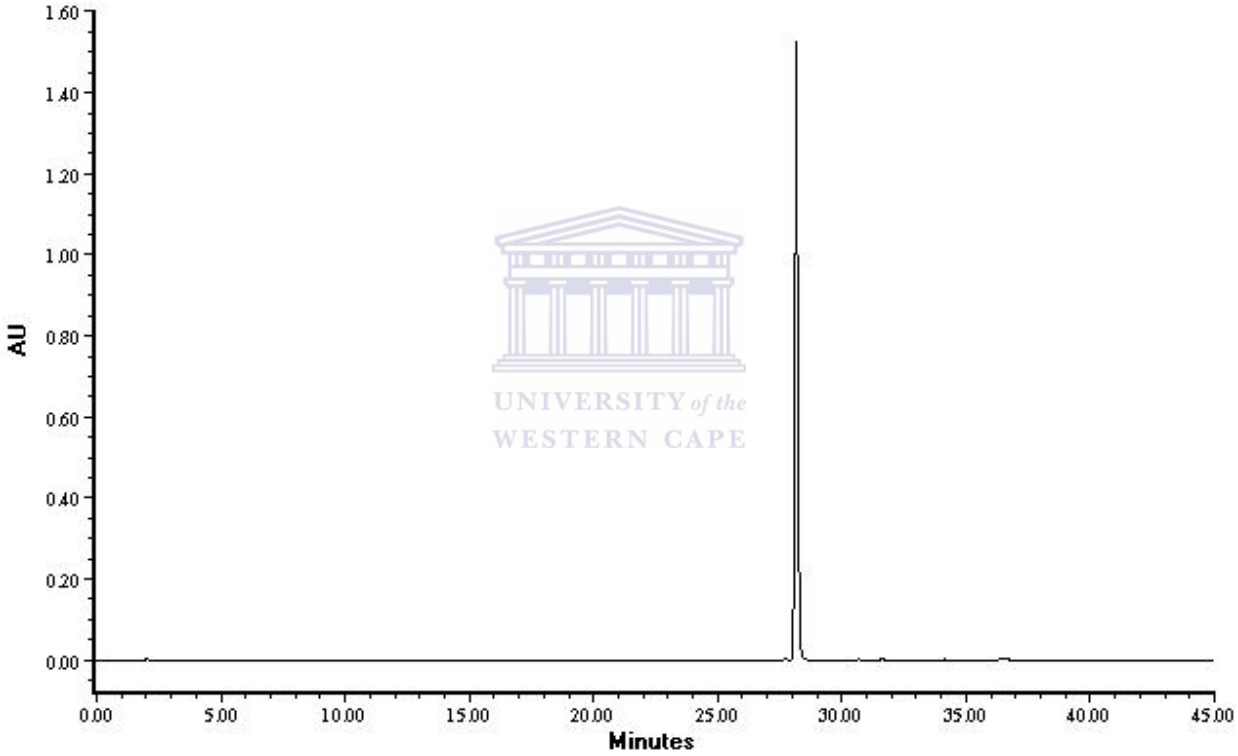
Trolox is a well-known non-phenolic antioxidant and was tested in this study in both the antioxidant assays in order to obtain a reference IC_{50} value (data not shown). It was noticeable that in both assays for the ranges tested (0.8 – 0.0008 mM and 20.2 - 2.53 µM final concentration in the reaction volume for the lipid peroxidation and $ABTS^{\bullet+}$ assays, respectively) the relationship between % inhibition and concentration followed a linear relationship whereas that of a typical dose-response curve is hyperbolic. It was therefore not possible to obtain a sigmoidal dose-response curve for the Trolox data obtained with the GraphPad Software and the IC_{50} values quoted in the text was calculated from the linear relationship.

Addendum 3B. Scanned image of silica 60 TLC glass plate developed in mobile phase comprising of $CHCl_3$:MeOH:H_2O:HAc:1100:720:160:20 to compare the Rf values of major rooibos flavonoids with that of epigallocatechin gallate (EGCG)



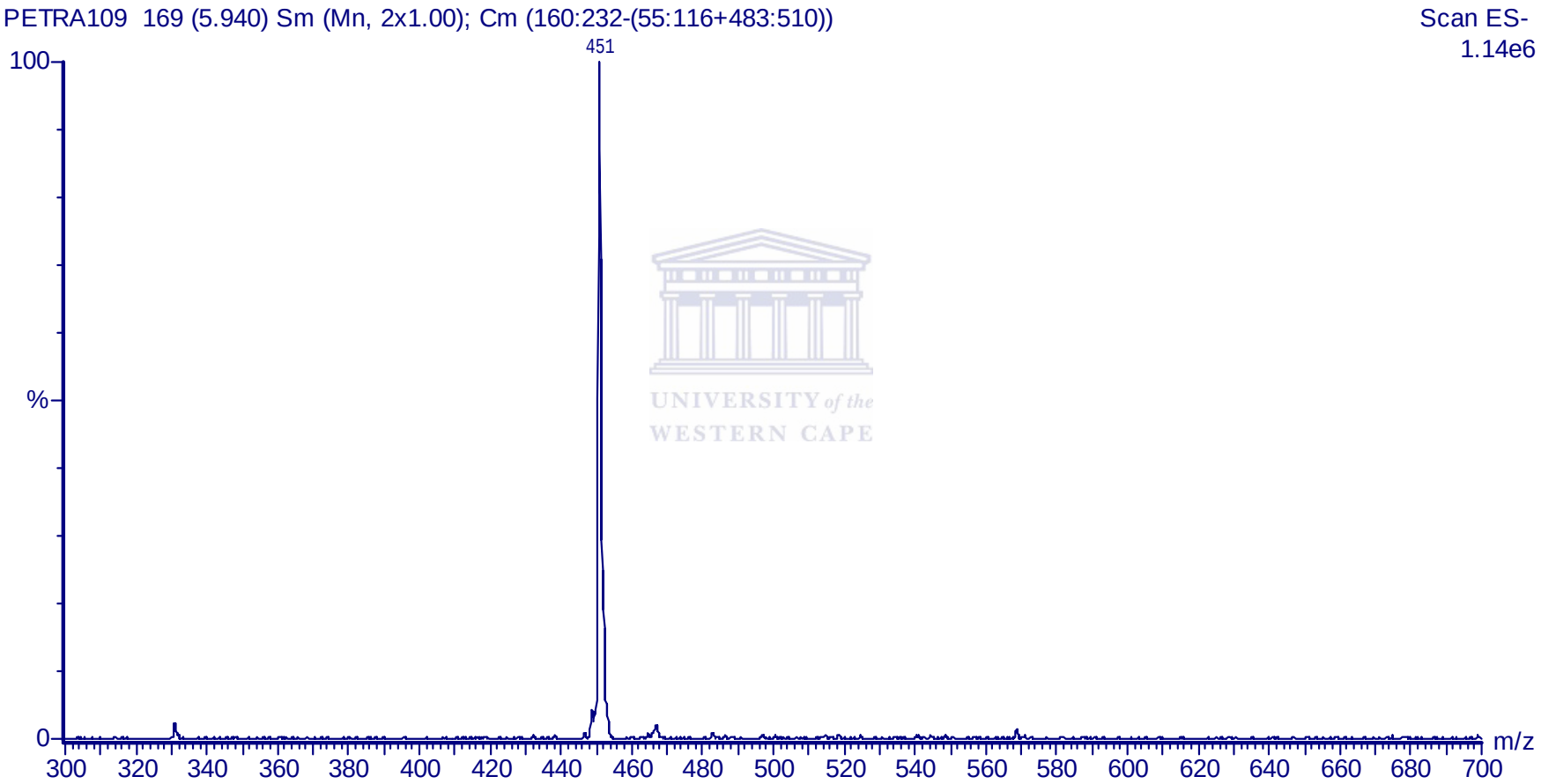
Key:

que: quercetin
hyp: hyperoside
rut: rutin
i-q: iso-quercitrin
not: nothofagin
i-vit: isovitexin
vit: vitexin
asp: aspalathin
or: orientin
i-or: iso-orientin
lut: luteolin
chr: chrysoeriol
cat: catechin
egcg: epigallocatechin gallate (from *Camellia sinensis*)



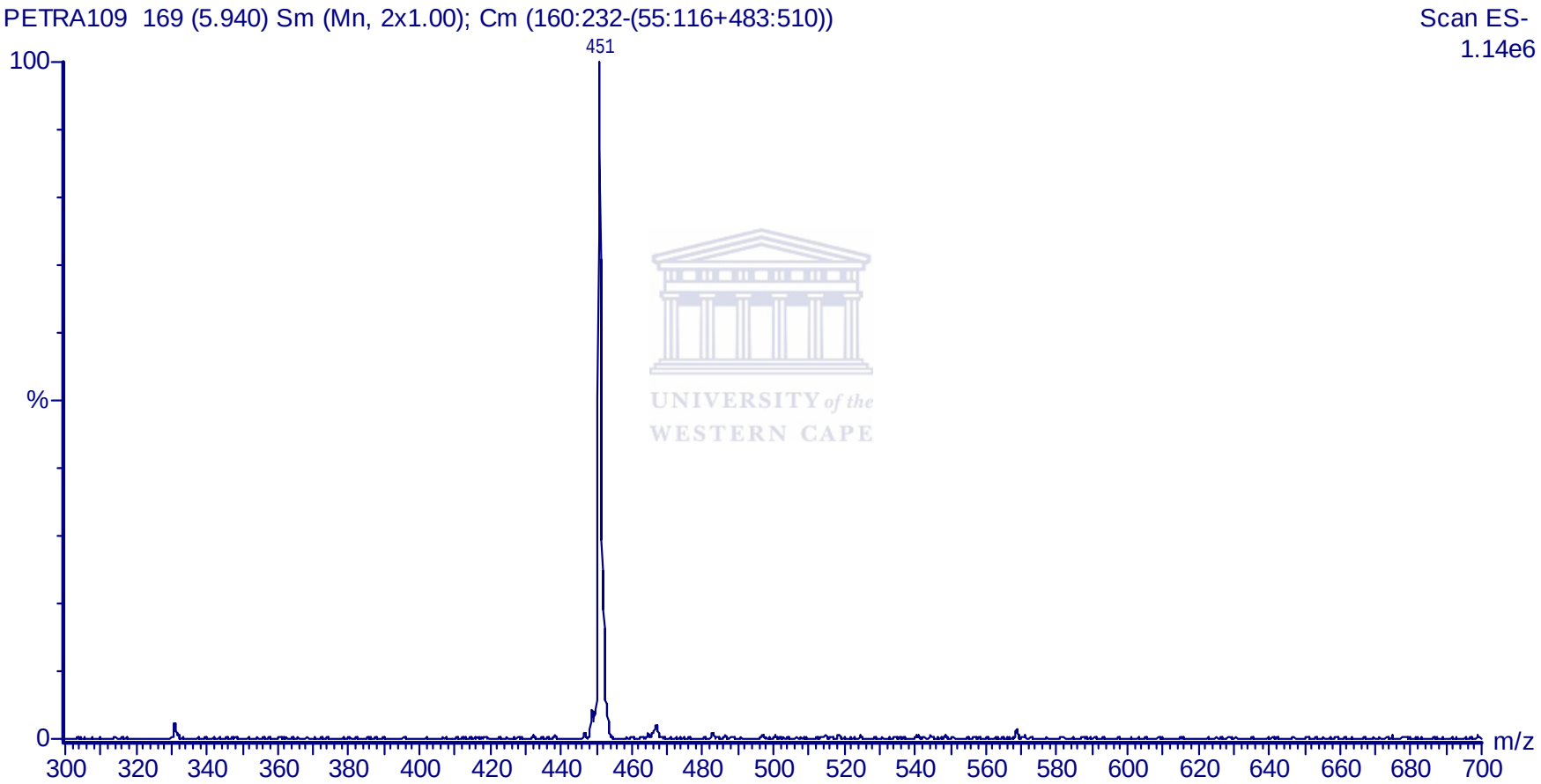
Addendum 3C. HPLC chromatogram showing purity of aspalathin at λ = 288 nm

09-Apr-2002
LCMS: Aspalathin (Promec); C18; 50% ACN 100µl/min..

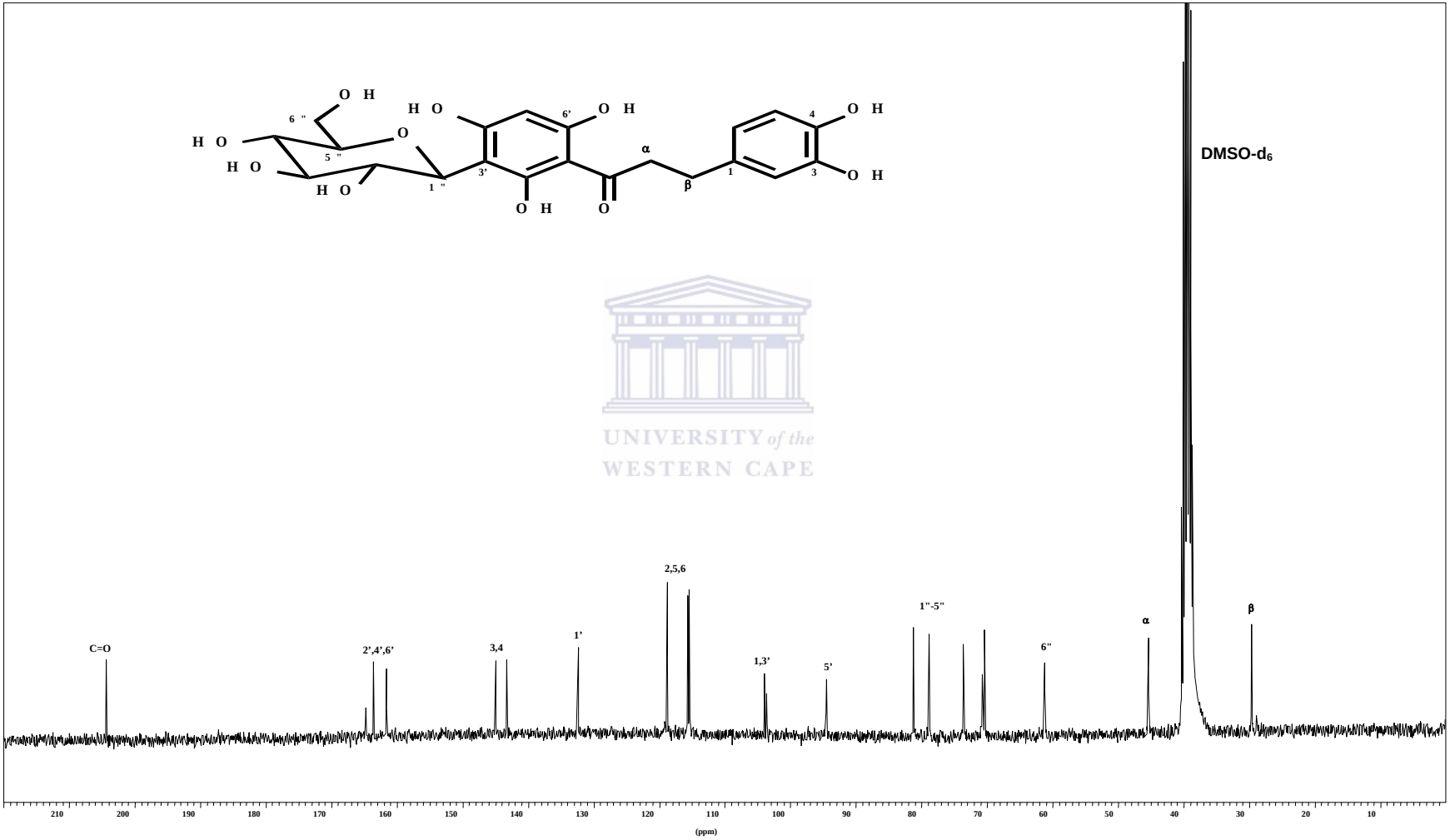


Addendum 3D. LCMS spectra of aspalathin.

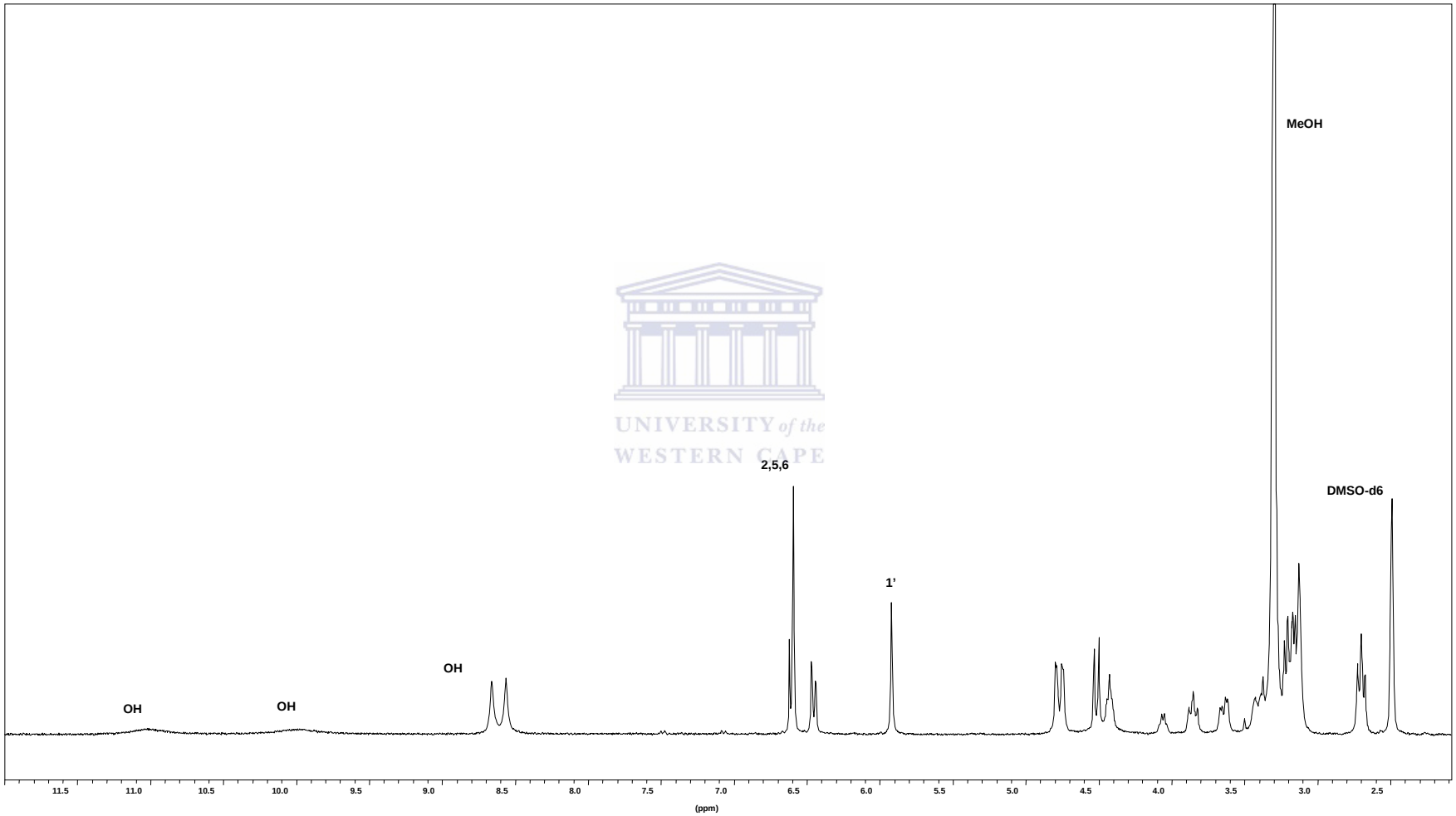
09-Apr-2002
LCMS: Aspalathin (Promec); C18; 50% ACN 100µl/min..



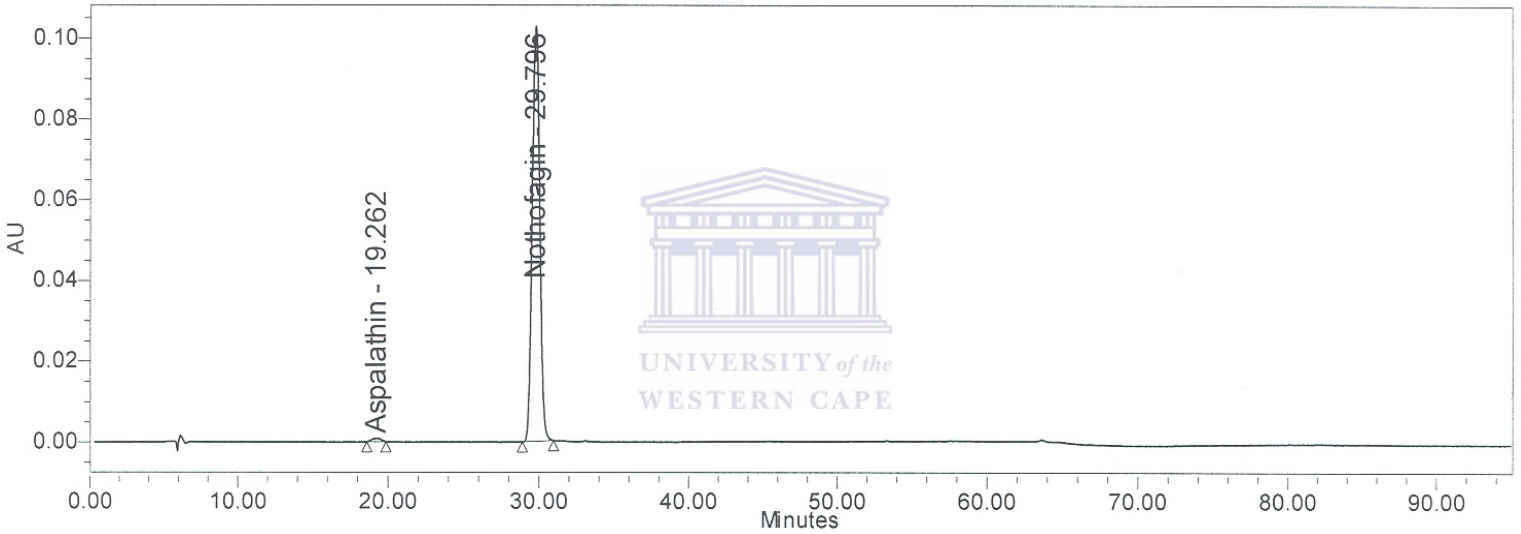
Addendum 3D. LCMS spectra of aspalathin.



Addendum 3E1. ^{13}C NMR spectrum of aspalathin (4.3 mg in DMSO-d_6, 24576 scans)

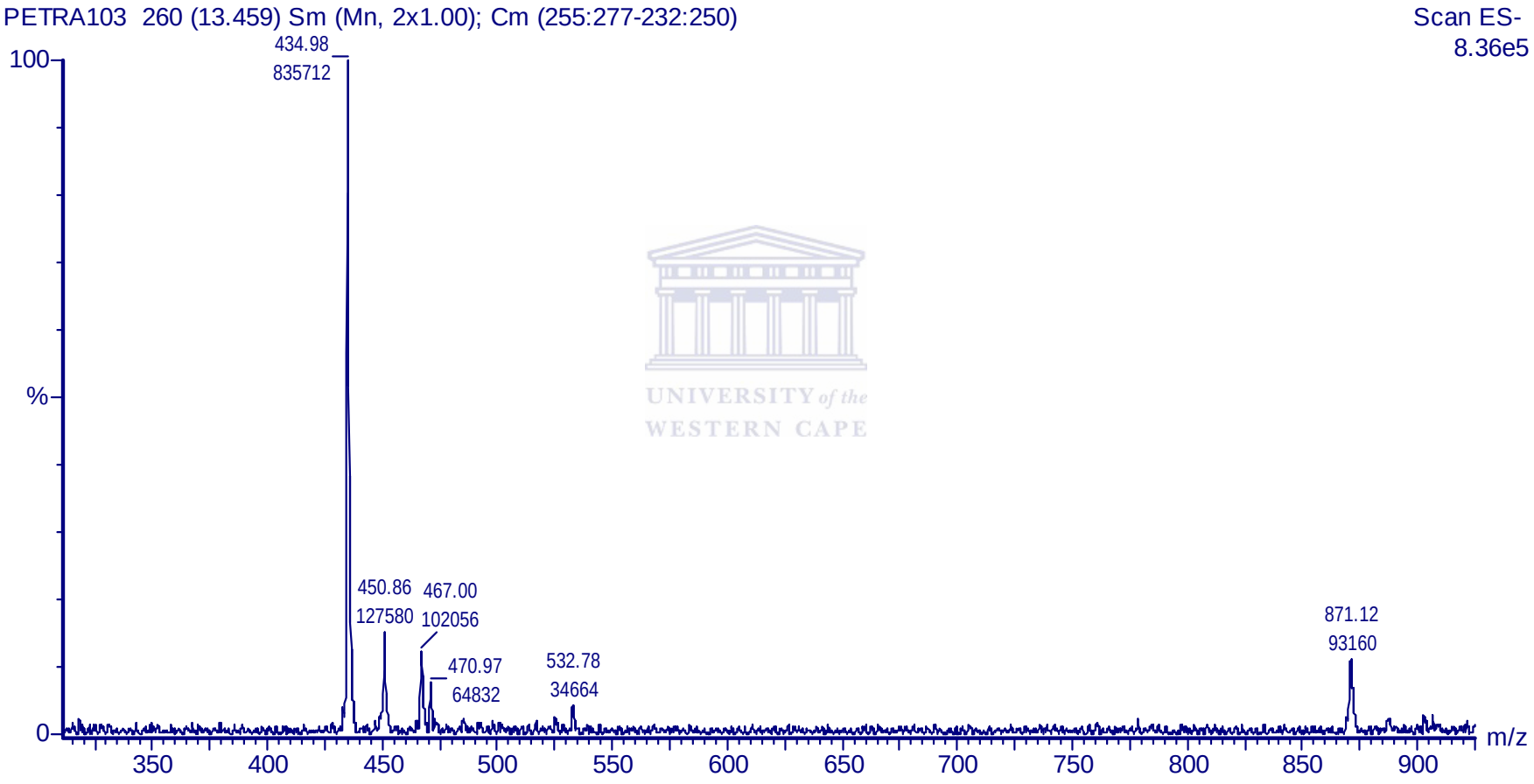


Addendum 3E2. ^{1}H NMR spectrum of aspalathin (4.3 mg in DMSO-d_6, 16 scans)



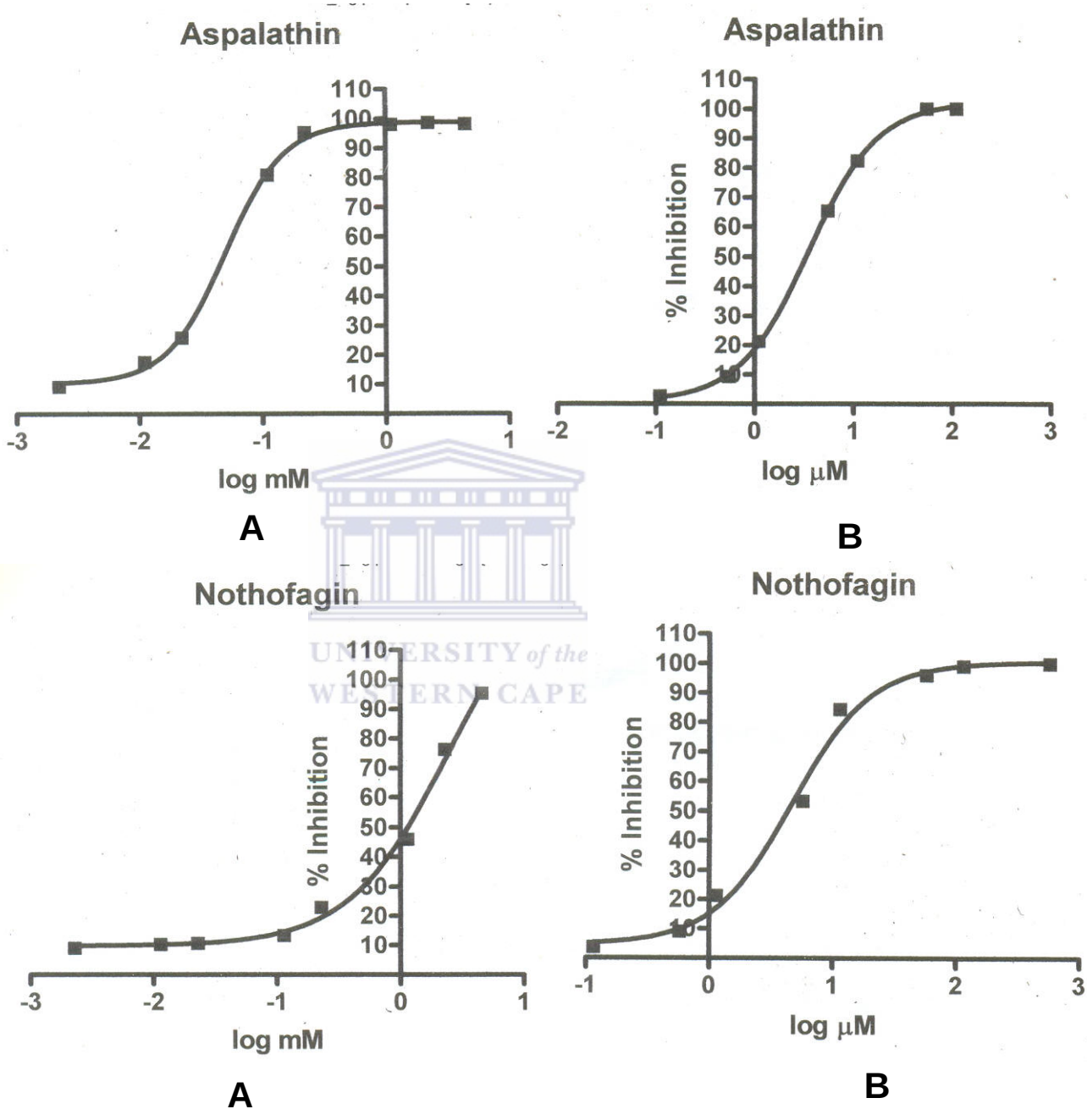
Addendum 3F. HPLC chromatogram showing purity of nothofagin at λ = 288 nm

09-Apr-2002
Notofagin in 50% ACN.



Addendum 3G. LCMS spectra of nothofagin.

ADDENDUM 4A. Sigmoidal dose response curves (variable slope) of aspalathin and nothofagin in the A. lipid peroxidation and B. $ABTS^{\bullet+}$ assays.



Note the absence of the upper plateau for nothofagin. This caused a wide 95% confidence interval for the IC_{50} value.