A Comparative Study on Protection of *Cyclopia spp.* (Honeybush), *Aspalathus linearis* (Rooibos) and *Camellia sinensis* Teas against Aflatoxin B_1 induced Mutagenesis in the *Salmonella* Mutagenicity Assay: Possible Mechanisms Involved

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Declaration

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

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Abstract

Antimutagenic activity of aqueous extracts of fermented and unfermented *Cyclopia spp.*, i.e. *C. intermedia, C. subternata, C. genistoides* and *C. sessiliflora* against metabolically activated aflatoxin B_1 (AFB_1) in the *Salmonella* mutagenicity assay with tester strain TA100, was compared to that of fermented and unfermented *Aspalathus linearis* (rooibos) and *Camellia sinensis* (black, oolong and green) teas. Possible mechanisms involved in *in vitro* antimutagenic activity of these teas were investigated, i.e. the stabilising effect of the unfermented *Cyclopia spp.,* unfermented rooibos and green tea on rat liver cytochrome P450 in the S9 fraction from Aroclor 1254 treated rats and their modulation of aniline-induced Type II difference spectra in the microsomal fraction. Inhibition of lipid peroxidation in rat liver S9, by the teas, was assessed to determine whether protection against lipid peroxidation may play a role in cytochrome P450 stability *in vitro*.

Correlation of the antimutagenic activity of the teas with their stabilising effect on cytochrome P450 and inhibition of lipid peroxidation, provided insight into possibly related mechanisms. Antimutagenic activity correlated weakly with a decreased stabilising effect of the teas on cytochrome P450 ($r = 0.411$, $P = 0.013$) and the inhibition of lipid peroxidation ($r = 0.475$, $P = 0.003$). Decreased stability of cytochrome P450 was associated with substantial lipid peroxidation occurring in rat liver S9. Effective inhibition of lipid peroxidation and stabilising of cytochrome P450 in S9 was evident in the presence of the teas, but no correlation ($r = 0.018$, $P = 0.915$) existed for the effect of unfermented teas on cytochrome P450 stability with inhibition of lipid peroxidation.

Black tea exhibited the highest protection against AFB_1-induced mutagenesis and fermented *C. intermedia* offered the least protection. "Fermentation" resulted in increased antimutagenic activity of *Camellia sinensis* and rooibos teas, while the antimutagenic activity of *Cyclopia spp.* decreased with fermentation except for *C. genistoides*. Unfermented teas significantly ($P < 0.05$) stabilised cytochrome P450, with rooibos more effective ($P < 0.05$) than green tea, but similar ($P < 0.05$) to C*yclopia spp.* Green tea demonstrated the highest inhibition of lipid peroxidation, while the inhibition exerted by rooibos was similar ($P > 0.05$) to unfermented *Cyclopia spp.*, except for *C. genistoides* exhibiting the least inhibition.

Total polyphenol, flavanol and flavonol/flavone contents of the respective teas were correlated with activity in terms of antimutagenicity, stabilising of cytochrome P450 and inhibition of lipid peroxidation. Antimutagenic activity of *Cyclopia spp.* correlated with its total

polyphenol ($r = 0.805$, $P < 0.0001$) and flavanol ($r = 0.653$, $P < 0.0001$) contents, while a weak negative correlation ($r = -0.456$, $P = 0.026$) was observed for the inhibition of lipid peroxidation by unfermented *Cyclopia spp.* with the flavonol/flavone content. Antimutagenicity of *Cyclopia spp.* correlated weakly ($r = 0.363$, $P = 0.012$) with its hesperidin content. Antimutagenic activity of rooibos tea correlated moderately ($r = 0.751$, $P < 0.005$) with its flavonol/flavone content and specifically the flavones orientin ($r = 0.674$, $P < 0.023$) and iso-orientin ($r = 0.728$, $P < 0.011$). A strong negative correlation ($r = -0.918$, $P < 0.0001$) of antimutagenicity of rooibos with its aspalathin content was observed. Antimutagenic activity of *Camellia sinensis* teas did not correlate with their total polyphenol, flavanol or flavonol/flavone contents. The flavanol content of green tea showed a good, but marginal ($P < 0.1$) correlation ($r = 0.824$, $P = 0.086$) with decreased cytochrome P450 stability.

The modulation of aniline-induced Type II binding to microsomal cytochrome P450 by green tea differed significantly ($P < 0.05$) from the modulation exhibited by rooibos and *Cyclopia spp.* Flavonoid glycosylation appeared to influence antimutagenic activity, stabilising of cytochrome P450 and modulation of substrate binding of selected phenolic compounds. The present study indicates that rooibos and *Cyclopia spp.* have *in vitro* antimutagenic activity against AFB_1, suggesting that consumption of these two herbal teas may have beneficial health effects. It is also suggested that stabilising of cytochrome P450 by tea, and interaction of tea constituents with cytochrome P450, may influence their *in vitro* antimutagenic activity.

Uittreksel

Antimutageniese aktiwiteit van waterekstrakte van gefermenteerde en ongefermenteerde *Cyclopia spp.* d.i. *C. intermedia*, *C. subternata* en *C. genistoides* teen metabolies geaktiveerde aflatoksien B_1 (AFB_1) in die *Salmonella* mutagenisiteitstoets, is vergelyk met dié van gefermenteerde en ongefermenteerde *Aspalathus linearis* (rooibos) en *Camellia sinensis* (swart, oolong en groen) tees. Meganismes wat moontlik betrokke is by die *in vitro* antimutageniese aktiwiteit van die tees is ondersoek, d.i. die stabiliseringseffek van ongefermenteerde *Cylopia spp.*, ongefermenteerde rooibos en groen tees op rotlewer sitochroom P450 vanaf Aroclor 1254 behandelde rotte in die S9 fraksie en hul modulasie van anilien-geïnduseerde Tipe II verskilspektra in die mikrosomale fraksie. Inhibisie van lipiedperoksidasie in rotlewer S9 deur die tees is bepaal om hul beskermende rol teen lipiedperoksidasie in die stabiliteit van sitochroom P450 te ondersoek.

Korrelasie van antimutageniese aktiwiteit met die stabiliseringseffek op sitochroom P450 en die inhibisie van lipiedperoksidasie deur die tees, het insig verskaf omtrent moontlike verwante meganismes. Antimutageniese aktiwiteit het ‘n swak korrelasie ($r = 0.411$, $P = 0.013$) met ‘n afname in die stabiliseringseffek van die tees op sitochroom P450, sowel as met die inhibisie van lipied peroksidasie ($r = 0.475$, $P = 0.003$) getoon. ‘n Afname in die stabiliteit van sitochroom P450 is in verband gebring met aansienlike lipiedperoksidasie in rotlewer S9. Die teenwoordigheid van tee het effektiewe inhibisie van lipiedperoksidasie en stabilisering van sitochroom P450 in S9 tot gevolg gehad. Daar was egter geen korrelasie ($r = 0.018$, $P = 0.915$) tussen die effek van ongefermenteerde tees op sitochroom P450 stabiliteit en die inhibisie van lipiedperoksidasie nie.

Swart tee het die meeste beskerming teen AFB_1-geïnduseerde mutagenisiteit getoon, en gefermenteerde *C. intermedia* het die minste beskerming gebied. “Fermentasie” het ‘n toename in die antimutageniese aktiwiteit van *Camellia sinensis* en rooibos tee tot gevolg gehad, terwyl die antimutageniese aktiwiteit van *Cyclopia spp.*, behalwe in die geval van *C. genistoides*, verlaag is deur fermentasie. Ongefermenteerde tees het ‘n beduidende ($P < 0.05$) stabiliseringseffek op sitochroom P450 gehad. Rooibos was meer effektief ($P < 0.05$) as groen tee, maar soortgelyk ($P > 0.05$) aan *Cyclopia spp.* Groen tee het die mees effektiewe inhibisie van lipiedperoksidasie getoon, terwyl die inhibisie deur rooibos gelykstaande ($P > 0.05$) was aan dié van ongefermenteerde *Cyclopia*, uitgesluit *C. genistoides* wat die minste inhibisie getoon het.

Die totale polifenol-, flavanol- en flavonol/flavooninhoud van die onderskeie tees is met hul aktiwiteit, in terme van antimutagenisiteit, stabilisering van sitochroom P450 en inhibisie van lipiedperoksidasie, gekorreleer. Antimutageniese aktiwiteit van *Cyclopia spp.* het met hul totale polifenol ($r = 0.805$, $P < 0.0001$) en flavanol ($r = 0.653$, $P < 0.0001$) inhoud gekorreleer, terwyl ‘n swak negatiewe korrelasie ($r = -0.456$, $P = 0.026$) vir die inhibisie van lipied peroksidasie met die flavonol/flavooninhoud van ongefermenteerde *Cyclopia spp* aangetoon is. Antimutagenisiteit van *Cyclopia spp.* het ‘n swak korrelasie ($r = 0.363$, $P = 0.012$) met hesperidininhoud getoon. Antimutagenisiteit van rooibos het ‘n matige korrelasie ($r = 0.751$, $P < 0.005$) met die flavonol/flavooninhoud, en spesifiek met die flavone, orientin ($r = 0.674$, $P = 0.023$) en iso-orientin ($r = 0.728$, $P = 0.011$) getoon. ‘n Sterk negatiewe korrelasie ($r = -0.918$, $P < 0.0001$) is verkry vir die antimutagenisiteit van rooibos met aspalatien inhoud. Antimutageniese aktiwiteit van *Camellia sinensis* tees het nie met hul totale polifenol-, flavanol- of flavonol-/flavooninhoud gekorreleer nie. Die flavanolinhoud van groen tee het ‘n goeie, maar marginale ($P < 0.1$) korrelasie ($r = 0.824$, $P = 0.086$) met ‘n afname in sitochroom P450 stabiliteit, getoon.

Die modulasie van anilien geïnduseerde Tipe II binding aan mikrosomale sitochroom P450 deur groen tee, het betekenisvol verskil van die modulasie deur rooibos en *Cyclopia spp.* Flavonoied glukosilering het die antimutageniese aktiwiteit, stabilisering van sitochroom P450 en modulasie van substraat binding van geselekteerde fenoliese verbindings, beïnvloed. Die huidige studie dui aan dat rooibos en *Cyclopia spp. in vitro* antimutageniese aktiwiteit teen AFB_1 het, wat aandui dat die verbruik van hierdie twee kruietees moontlike voordele vir menslike gesondheid kan inhou. Stabilisering van sitochroom P450 deur die tees en interaksie van verbindings in tee met sitochroom P450, beïnvloed waarskeinlik die *in vitro* antimutageniese aktiwiteit van tee.

I wish to dedicate this thesis to Henk

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Contents

The language and style of this thesis is in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has therefore been unavoidable.

Chapter 1
Introduction

An excess of twenty million people living with cancer, ten million new cases worldwide and six million cancer related deaths each year, clearly illustrate the increased cancer burden in all regions of the world (WHO/UICC, 2003). An anticipated increase to ten million cancer related deaths per year by 2020 makes the importance of research investigating treatment and prevention of this disease unambiguous.

Nutrition and diet are regarded as major factors contributing to human carcinogenesis (Sugimura, 2000), but contrarily preventive effects of natural occurring dietary elements against tumour induction and growth have been demonstrated (Wattenberg, 1996; Kim & Masuda, 1997; Siess *et al*., 1997; De Marini, 1998; Surh, 1999; Ferguson *et al*., 2004). Such dietary compounds are referred to as chemopreventive agents, with chemoprevention being the administration of non-toxic compounds, preferably originating from dietary constituents such as fruits, vegetables and beverages, to lower the rate of cancer incidence (Tanaka, 1997). The eminent fact that fruit and vegetable intake is associated with a reduced risk of cancer (Wargovich, 1997; Siess *et al*., 1997; Ames, 1998) has mainly been attributed to beta-carotene and ascorbate, but it is thought that phenolic constituents may also play a role (Heim *et al*., 2002). The protective effect of the polyphenol group, flavonoids, against degenerative diseases such as cancer and coronary heart disease, is partly attributed to antioxidant activity (Rice-Evans *et al*., 1997; Heim *et al*., 2002) resulting in prevention of processes involved in diseases, such as lipid peroxidation and oxidative DNA damage (Collins, 1999). Lipid peroxidation contributes to a number of pathological conditions including cancer, inflammatory processes, atherosclerosis, toxic injury by xenobiotics and ischemic-reperfusion damage (Dargel, 1992).

Health issues have become increasingly important to consumers (Simrany, 1998) and have resulted in a thriving market for products with medicinal value and a growing demand for natural products (Pettigrew, 2001). Herewith a dramatic rise in the popularity and credibility of tea as a health beverage has been experienced worldwide. Tea, mainly used as descriptor for three kinds of infusions produced from *Camellia sinensis*, i.e. black (fermented), oolong (semi-fermented) and green tea (unfermented), is one of the most popular beverages in the world (Chu, 1997) and contains substantial amounts of polyphenols (Balentine *et al*., 1997; Wiseman *et al*., 1997; Beecher *et al.,* 1999). Chemopreventive effects of tea polyphenols have been demonstrated in different bioassay systems (Kada *et al*., 1985; Wang *et al*., 1989; Mukhtar *et al*., 1992;

Apostolides *et al*., 1996; Weisburger *et al*., 1996; Chen & Yen, 1997; Wargovich, 1997; Kuroda & Hara, 1999; Suganuma *et al*., 1999). Numerous reviews summarise the chemopreventive activity of tea produced from *Camellia sinensis* (Yang & Wang, 1993; Lin & Liang, 2000; Gupta *et al*., 2002; Lambert & Yang, 2003) and anticarcinogenic activity of tea is still the subject of many studies (Siess *et al*., 2000; Catterall *et al*., 2003; Park & Surh, 2004; Way *et al*., 2004).

Herbal infusions serve as an alternative to black and green teas for consumers who prefer caffeine free beverages (Peet, 1995) and increased consumption of herbal and fruit infusions is attributed to consumer perception that it is healthy, pure and natural (Beerbaum, 1997). Two indigenous South African herbal teas, *Aspalathus linearis* (Burm. Fil) R. Dahlgr. *spp. linearis*, known as rooibos and *Cyclopia spp.*, known as honeybush tea, are consumed locally and abroad as health beverages (Joubert & Ferreira, 1996; Snyman, 2000; De Villiers, 2002, De Villiers, 2004). A complex mixture of polyphenolic compounds has been isolated from rooibos (Rabe *et al*., 1994; Ferreira *et al*., 1995; Marais *et al*., 2000; Bramati *et al*., 2002) and honeybush tea (De Nysshen *et al.,*1996; Ferreira *et al*., 1998, Kamara *et al*., 2003; Kamara *et al*., 2004), which differs significantly from each other and that of *Camellia sinensis* teas (Balentine *et al*., 1997). Several health properties, mostly based on anecdotal evidence, are associated with the consumption of rooibos (Joubert & Ferreira, 1996) and honeybush teas (Watt & Breyer-Brandwijk, 1932; Morton, 1983).

Rooibos and honeybush teas possess antimutagenic (Marnewick *et al*., 2000; Standley *et al*.,2001; Richards, 2002) and antioxidant activity (Von Gadow *et al*., 1997; Hubbe & Joubert, 2000a; Hubbe & Joubert, 2000b, Richards, 2002). The protection of honeybush tea, i.e. *C. intermedia, C. sessiliflora, C. genistoides* and *C. subternata*, against lipid peroxidation and decreased activity with fermentation (oxidation during processing), except for *C. genistoides*, have been demonstrated (Hubbe, 2000; Richard, 2002). Inhibition of lipid peroxidation by fermented and unfermented rooibos tea has been shown in different model lipid systems, including micelles and emulsions (Winterton, 1999).

Standley (1999) demonstrated antimutagenic activity of rooibos tea for the first time in the *Salmonella* mutagenicity assay, in the presence of metabolically activated 2-acetylaminofluerene (2-AAF). Antimutagenic activity of rooibos tea was confirmed by Marnewick *et al.* (2000) who reported the protective effect of aqueous extracts of fermented and unfermented rooibos and honeybush tea (*C. intermedia*) against 2-AAF and aflatoxin B_1 (AFB_1) induced mutagenesis in the *Salmonella* mutagenicity assay. In a subsequent study it was demonstrated that aqueous extracts of four species of fermented and unfermented honeybush i.e. *C. intermedia, C.*

sessiliflora, C. genistoides and *C. subternata* had antimutagenic activity against 2-AAF, with the exception of unfermented *C. genistoides* which enhanced mutagenicity of 2-AAF in the *Salmonella* mutagenicity assay (Richards, 2002).

Recent studies on rooibos and honeybush teas reported that they affect the induction of phase II hepatic drug metabolising enzymes and increase the antioxidant status of the liver, which may partly explain antimutagenic activity of these beverages (Marnewick *et al*., 2003). A study investigating the *ex vivo* modulating effects of sub-cellular hepatic fractions of rats treated with fermented and unfermented rooibos and honeybush (*C. intermedia*) teas demonstrated that unfermented extracts resulted in significant protection against 2-AAF induced mutagenesis in the *Salmonella* mutagenicity assay (Marnewick *et al*., 2004). Activation of AFB_1 was significantly reduced by microsomal fractions prepared from livers of rats treated with fermented and unfermented rooibos and unfermented honeybush (*C. intermedia*) teas (Marnewick *et al*., 2004).

Possible mechanisms through which tea polyphenols exert their antimutagenic activity include inhibition of promutagen activation, inactivation of mutagens and carcinogens, blocking and scavenging of reactive molecules, modulation of DNA replication or repair and inhibition of promotion (De Flora, 1998; Kuroda & Hara, 1999). It has been proposed that inhibition of promutagen activation involves interaction of polyphenols with the cytochrome P450 activation system which results in reduced formation of ultimate carcinogenic metabolites (Das *et al*., 1985; Das *et al*., 1987; Wang *et al*., 1987; Wang *et al*., 1989). Cytochrome P450, a large family of hemoproteins, is not only central to the detoxification of a remarkable number of foreign hydrophobic compounds, including many therapeutic drugs and environmental pollutants (Kamataki, 1993; Omura, 1999), but also to the activation of chemical carcinogens (Omura, 1993). Inhibition of cytochrome P450-dependent monooxygenase activities by green tea polyphenols have been demonstrated through changes in difference spectra upon addition of green tea extracts and polyphenols to rat liver microsomes (Wang *et al*., 1987). It was postulated that the observed inhibition of enzyme activities and alteration in difference spectra is in part due to binding of the phenolic groups to the catalytic sites of cytochrome P450, which consequently lead to alteration in the structure and function of cytochrome P450 (Wang *et al*., 1987). This in turn may result in altered rates and differential pathways of metabolism of mutagens and carcinogens (Wang *et al*., 1989). Information on the antimutagenic activity of honeybush tea is limited and mechanisms involved in this activity in the *Salmonella* mutagenicity assay has to date not been investigated.

This study was aimed at expanding the knowledge on antimutagenic activity of honeybush tea and to gain perspective on this aspect through comparison to rooibos and tea produced from the *Camellia sinensis* plant (black, oolong and green teas). Antimutagenicity of freeze-dried aqueous extracts prepared from fermented and unfermented honeybush and rooibos as well as *Camellia sinensis* teas and the major monomeric phenolic compounds present in the respective teas, was assessed in the *Salmonella* mutagenicity assay. AFB_1 was selected as carcinogen since it is known as a potent hepatotoxin and hepatocarcinogen (Shen *et al*., 1995; IARC, 2002; Wogan *et al*., 2004). Possible mechanisms involved in antimutagenesis in the *Salmonella* mutagenicity assay were investigated by assessing (i) the stabilising of cytochrome P450 and inhibition of lipid peroxidation at 37°C *in vitro* and (ii) the modulation of aniline-induced Type II substrate binding to cytochrome P450, by freeze-dried aqueous extracts prepared from the unfermented teas and major monomeric phenolic compounds present in the respective teas.

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Chapter 2

Literature Review

A. Introduction

Dietary habits have in recent years been regarded as one of the significant causative factors that affect human health (DeMarini, 1998), and it is known that many types of cancer relate to diet (Bailey & Williams, 1994; Ames *et al*., 1995; Katiyar & Mukhtar, 1997; Ferguson *et al*., 2004). On the contrary, the human diet is not only a source of macronutrients, micronutrients, mutagens and/or carcinogens, but also contains a number of constituents that inhibit carcinogenesis known as antimutagens and anticarcinogens (Bailey & Williams, 1994; Karakya & Kavas, 1999; Surh, 1999). Identification and use of such cancer preventive agents contained in the human diet have become an important issue in public health-related research (Gerhäuser *et al*., 2003).

Natural dietary inhibitors of mutagenesis and carcinogenesis hold great promise to reduce human disease (Bailey & Williams, 1994). Plant constituents such as flavonoids and isoflavonoids occur frequently in the human diet and exhibit several biological properties that may account for antimutagenic and anticarcinogenic activity (Surh, 1999; Siess *et al*., 2000; Birt *et al*., 2001).

This literature review consists of a brief discussion of carcinogenesis, the sources of mutagens/carcinogens and carcinogen metabolism with special reference to the role of the drug metabolising enzyme, cytochrome P450 in carcinogen metabolism. Anticarcinogenesis, antimutagenesis and the mechanisms concerned, as well as sources of antimutagens will also be discussed. An overview of the composition of *Cyclopia spp., Aspalathus linearis* and *Camellia sinensis* as well as the role of tea polyphenols as antimutagens and their interaction with cytochrome P450 will be given.

B. Carcinogenesis

Cancer

Cancer can principally be described as an uncontrolled proliferation of abnormal cells that in due course results in death, a phenomena that displays an increased incidence with age (Tanaka, 1997). It is a genetic disease caused by the accumulation of damage to DNA, which causes the tumour cell to become insensitive to growth controlling signals from the surrounding cellular environment (Sarasin, 2003). Greek physicians recognised the existence of cancer and the

distinction between benign and malignant tumours (Johnson, 2003). Tumours are described as any focal accumulation of cells beyond the numbers required for the development, repair or function of a tissue. Benign tumours are characterised by slow growth and the ability to retain the specialisation and spatial localization of the tissue it originated from (Johnson, 2003). Malignant tumours or cells grow much faster, lose differentiation and have a tendency to invade surrounding tissues and migrate to other organs to form secondary tumours or metastases. Carcinomas are malignant tumours resulting from epithelial cells while sarcomas are formed from connective or mesenchymal cells (Johnson, 2003).

Primary sites for adult cancers include the lung, colon and uterus which are carcinomas of epithelial origin and indicate a selective vulnerability of these tissues to carcinogenic insult as a result of frequent exposure to external environment (Surh, 1999). The underlying mechanisms of carcinogenesis is not clearly understood, nevertheless it is well known to be characterised by a complex process that involves a series of individual steps (Tanaka, 1997; Surh, 1999; Johnson, 2003).

The multi-step cancer process

A simple experimental model of carcinogenesis based on various experimental data, represents it as a three-stage process (Armitage & Doll, 1954; Pitot, 1989; Pitot & Dragan, 1994; Siess *et al*., 1997; Kuroda & Hara, 1999; Surh, 1999).

The first stage, the **initiation step** (Fig. 1), occurs after incorporation, distribution and metabolism of carcinogenic agents within the body. The term initiation originated from this stage, which involves the initial alteration in individual cells. Cells acquire mutations through damage to the genomic DNA as a result of exposure to a carcinogen, which is then passed on to a clone of daughter cells, through repeated division. The initiation step involves changes at the genetic level requiring cell division for fixation. This step is irreversible.

The subsequent stage, **promotion** (Fig. 1) is reversible and results in clonal expansion of the initiated (damaged) cells and the construction of actively proliferating preneoplastic cells. These cells are less responsive to cellular signals for maintainance of a normal state and normal growth. During promotion additional mutations to proto-oncogenes and tumour-suppressor genes occur and lead to progressive loss of differentiation and orderly growth. Proto-oncogenes are genes expressed to facilitate increased cellular proliferation at critical stages in the development or function of a tissue.

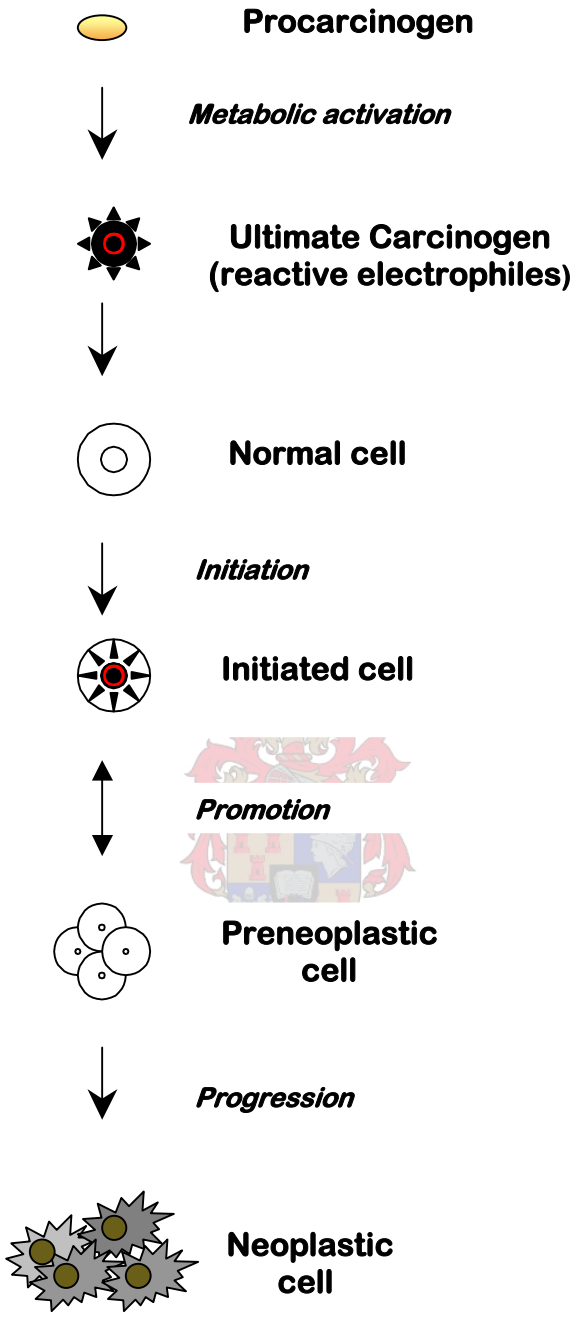


Figure 1 The multi-step process involved in carcinogenesis (modified from Surh, 1999).

When activated inappropriately within the mammalian genome they are referred to as oncogenes and result in increased rates of cellular proliferation. Mutations in tumour expression genes can result in conditions that favour tumour growth. An example would be the tumour suppressor gene p53 which functions as a regulator of cell proliferation and as a mediator of programmed cell death (apoptosis) in response to unrepaired DNA damage. Mutations rendering this gene non-functional, result in uncontrolled proliferation of cells containing DNA damage, instead of undergoing apoptosis.

The final stage, **progression** (Fig 1), is marked by the formation of neoplastic cells or a neoplasm, which is defined as a hereditary alteration in the growth patern of a cell. Neoplasms are characterised by an increased growth rate, invasiveness, potential and actual metastases and hormonal responsiveness. This stage is irreversible and characterised by verifiable alterations in the cell genome, which also distinguish it from the promotion stage.

Mutations and Cancer

Mutations are the alterations in DNA structure that produce permanent changes in the genetic information encoded in the DNA (Nelson & Cox, 2000). There is considerable evidence that gene and chromosomal mutations are important factors in carcinogenesis (Ramel, 1986) and the correlation between carcinogenicity and mutagenicity is estimated at 83% (Ames & McCann, 1981). The first step in mutagenesis involves the formation of primary lesions, which are chemical changes, such as damaged bases and chromosome breaks, generated in DNA by mutagens (Wallace *et al*., 1996; Ames *et al*., 1995). Most primary lesions are temporary and quickly eliminated from the gene by highly efficient DNA repair processes, but those improperly repaired or not repaired, become mutations (Ames *et al*., 1995).

Endogenous DNA damage is relatively high, while exogenous mutagens increase the number of lesions over the background rate of endogenous lesions (Ames *et al*., 1995). Mutagens have a more profound effect if it increases the rate of cell division in cells that are not discarded. Stem cells appear to be the important cells in cancer, since they are not discarded like the daughter cells and an increased division rate of stem cells therefore increases the probability of mutations. Several diverse factors are responsible for this increase of cell division and include elevated levels of particular hormones (Henderson *et al*., 1982), excess calories (Ames *et al*., 1995), chronic infections and inflammation (Ohshima & Bartsch, 1994), or certain doses of chemicals (Moalli *et al.*, 1987).

There are fundamentally three levels of mutational change: (1) **point** (gene) mutations, where a single base is modified, or one or relatively few bases are inserted or deleted; (2) **chromosomal mutations** which include large deletions or rearrangements of DNA, as chromosome breaks or rearrangements; or (3) gain or loss of whole chromosomes (Wallace *et al*., 1996; Mortelmans & Zeiger, 2000). Point mutations and chromosomal mutations are the result of unrepaired or improperly repaired damage to DNA (Wallace *et al*., 1996).

C. Sources of mutagens/carcinogens

Although investigation into the causes of cancer dates back approximately 150 years, rapid progress has only been made during the last 40 years (Weisburger, 2001). Cancer rates vary dramatically in regions and populations around the world with a profound difference between developed and developing countries with industrialisation, urbanisation and migration contributing to such variation (WCRF, AICR, 1997). This strongly indicates that cancer rates are influenced by environmental factors.

Major risk factors for cancer comprise **exogenous factors** such as diet, environment (radiation and industrial pollutants), tobacco use, other lifestyle factors such as regular alcohol consumption, low levels of physical activity, intense sun exposure and viruses (Bailey & Williams, 1994; Ames *et al*., 1995; Surh, 1999; Sugimura, 2000; Ferguson *et al*., 2004) and **endogenous damage** caused by oxidant by-products of normal metabolism (Ames *et al*., 1995; Newcomb & Loeb, 1998; Collins, 1999). It is estimated that as little as 2% of cancer cases are possibly the result of **inherent genetic alterations** (Knudson, 1977).

Exogenous Risk Factors in Cancer

Diet and lifestyle

In a 1981 review on avoidable risk factors of cancer in the United States, Doll & Peto (1981) attributed roughly 35% of cancer deaths to dietary factors, although the probable contribution of diet ranged from 10 to 70%. There has been a considerable increase in data available on the association between diet and cancer, which generally supports an earlier estimate of between 20 and 40% of cancer deaths being the result of dietary habits (Willett, 1995). Although epidemiologic studies do not produce hard experimental data that prove specific cancer-causative factors, it provides associative hypotheses or "risk factors" (Bailey & Williams, 1994).

Several **foodborne compounds** that are able to cause mutations, transform cell growth characteristics and increase cancer rates in experimental animals, have been identified from epidemiological suspicious foods such as broiled or salty fish, fermented vegetables, and moldy nuts and grains (Bailey & Williams, 1994). These chemicals are of potential concern to human health and can be found in food supply from a variety of sources. High meat and saturated fat consumption, increasing rates of obesity, and regular consumption of alcohol and tobacco are additional life style factors thought to indirectly enhance the probability of mutation (Ferguson *et al.*,2004). Infections by bacteria, parasites or viruses and tissue inflammation such as gastritis, hepatitis and colitis are also recognised risk factors for human cancers (Lewis & Adams, 1987; Ohshima & Bartsch, 1994). An overview of some of the known sources of diet-related carcinogenic compounds follows.

Mycotoxins

More than 300 secondary metabolites from filamentous fungi have been classified as mycotoxins to date. They are primarily produced by the moulds from the genera *Aspergillus, Penicillium,* and *Fusarium* (Bünger *et al*., 2004). Mycotoxins are produced in food as a result of fungal growth and cause a toxic response, known as mycotoxicosis, when ingested by higher vertebrates and animals (Sweeney & Dobson, 1998). Human ingestion of mycotoxins occurs mainly through plant-based foods and the residues and metabolites present in animal-derived foods. The range of toxic effects induced by mycotoxins include deterioration of liver or kidney function, neurotoxic effects, interference with protein synthesis and effects ranging from skin sensitivity to extreme immunodeficiency (Sweeney & Dobson, 1998). *Penicillium* and *Aspergillus* species occur frequently as contaminants of foods and other commodities during drying and subsequent storage, while *Fusarium* species are destructive plant pathogens that produce mycotoxins before, or immediately post harvest (Sweeney & Dobson, 1998). The presence and production of mycotoxins in foods or feeds are mainly influenced by storage, environmental and ecological conditions (Hussein & Brasel, 2001).

Mycotoxins of greatest public health and agroeconomic significance resulting in significant annual worldwide losses in human health, animal health, and condemned agricultural products, include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids (Hussein & Brasel, 2001).

Aflatoxins are the mycotoxins of greatest significance in foods and feeds and are mainly produced by *Aspergillus flavus*, *A. parasiticus* and *A. nominus* (Sweeney & Dobson, 1998).

Aflatoxins are the most thoroughly studied of this class of foodborne carcinogens and are thought to represent the greatest threat to human health of all the mycotoxins due to correlatory evidence that aflatoxin B_1 (AFB_1) is a liver carcinogen in human populations (Wogan, 1992; Bailey & Williams, 1994; Wogan *et al*., 2004). Exposure occurs through dietary intake of contaminated food sources such as rice, corn, sorghum, cereals, peanuts and soybeans (Prescott *et al*., 1999). AFB_1 has been found to be consistently genotoxic and produces DNA adducts in humans and animals and was classified as a group 1 carcinogen, substances that can cause cancer in humans, by the International Agency for Research on Cancer (IARC, 1993 & 2002). It produces DNA damage, gene mutation and chromosomal anomalies in human and animal cell cultures. The levels of aflatoxins in foods can vary from undetectable to as high as 12 000 ppb (Bailey & Williams, 1994). The maximum legal level of total aflatoxins in foodstuffs in South Africa is 10 micrograms per kilogram, of which 5 micrograms per kilogram (parts per billion) may be AFB_1 according to the regulations governing tolerances for fungus-produced toxins in foodstuffs (Government Notice No. R1124 of 8 August 2003; Anonymous, 2003).

Nitrosamines and nitrosamides (N-Nitroso compounds)

Numerous chemical carcinogens have been detected in the human diet, but N-nitroso compounds are among the most recent and are distinguished by their potentency in inducing mutations (Lijinsky, 1999). More than 90% of the approximately 300 different N-nitrosocompounds evaluated have been found to be carcinogenic (Bailey *et al*., 1991). These compounds are seldom present due to deliberate (if coincidental) addition and human exposure occurs through three main routes: (i) exogenous levels in foods (Krul *et al*., 2004); (ii) tobacco smoke (Bailey & Williams, 1991) and (iii) endogenous formation in the acidic environment of the stomach (Krul *et al*., 2004; Mukherjee *et al*., 2004). N-nitroso compounds are formed through interaction of nitrite and nitrogen oxide with secondary and tertiary amino compounds and are found almost exclusively in foods that contain nitrite or have been exposed to nitrogen oxides (Lijinsky, 1999).

Nitrosamides are unstable and therefore exogenous N-nitroso compounds in foods occur as nitrosamines, which are a broad class of compounds formed from the nitrosation of substituted amides, ureas, carbamates, and guanidines (Hotchkess, 1989). Nitrosamines are also formed from nitrogen oxides present in gas flames or from other burning used to cook food (Ames *et al*., 1995). Nitrosamides are direct-acting carcinogens (discussion on classes of carcinogens follows) and activation occurs by spontaneous hydrolysis (Bailey & Williams, 1994).

Food sources that contain N-nitroso compounds include cured meats, especially bacon, and increased levels of these compounds result when such foods are cooked, with concentrations of up to 100 μg kg^{-1} found (Lijinsky, 1999). Much higher concentrations of nitrosodimethylamine, but lower levels of other nitrosamines, have been found in Japanese smoked and cured fish (more than 100 μg kg^{-1}) with as much as 70 μg L^{-1} nitrosodimethylamine reported in some types of German beer, although usual levels are much lower (10 or 5 μg L^{-1}) (Lijinsky, 1999).

Nitrous anhydride commonly serves as a nitrosating agent and is formed from sodium nitrite added to several foods as a preservative or colour enhancer (Bailey & Williams, 1994). The stomach is a very favourable environment for the *in vivo* formation of endogenous N-nitroso compounds from nitrosating agents reacting with a number of dietary precursors. High nitrate levels in processed foods are regarded as a potential risk factor (Mukherjee *et al*., 2004) due to the formation of N-nitroso compounds *in vivo*, as these chemicals induce tumours in various organs including the liver, lungs, kidneys, bladder, pancreas and tongue (Lijinsky, 1999).

Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarcons (PAHs) are formed during incomplete combustion of organic material, especially coal and petroleum and are found ubiquitously in our environment (Dipple & Bigger, 1991). These compounds are fused aromatic ring systems of which many are not carcinogenic. Benzo[a]pyrene (B[a]P), a five-ring PAH, is a potent carcinogen and one of the most extensively studied of this group of foodborne carcinogens. PAHs are found in concentrations of up to 200 ppb in charcoal-broiled meats and are deposited on the surface of the meat during cooking (Lijinsky, 1991). Higher PAH levels are associated with meat with a higher fat content and is attributed to dripping of fat onto the charcoal and consequent pyrrolysis and deposition by smoke. PAH levels can be significantly reduced by broiling meat with the heat source above the meat.

Wood smoke also contains at least 100 PAHs and their alkylated derivatives of which many are carcinogenic (Stolyhwo & Sikorski, 2004). B[a]P is regarded as a marker of the carcinogenic PAH in smoke and smoked fish. Low levels of PAHs found in certain foods originate from environmental exposures such as fish caught in urban waterways, from contact with coal or petroleum products and fumes or atmospheric deposition from burning of gasoline or diesel engines, burning of fuels, and factory outputs (Bailey & Williams, 1994). Although PAHs differ in carcinogenic potency, even those that are regarded as non- carcinogenic may function in living

organisms as synergists and increase the carcinogenicity of other PAHs (Stolyhwo & Sikorski, 2004).

Amino acid pyrolysis products: heterocyclic amines

Risk factors linked to the consumption of animal protein could be due to generation of certain heterocyclic aromatic amines during cooking of proteinaceous food such as meat and fish (Edenharder *et al*., 1993). Interest in and the study of heterocyclic amines (HA) and their modulators as possible etiological agents in the development of human cancer have increased dramatically since a report in 1939 describing cancer producing substances in roasted food (Dashwood, 2002). Human exposure to heterocyclic amines occurs mainly through the consumptoin of meat cooked at a high temperature and are formed when creatine or creatinine and amino acids in meat pyrolyze (Sinha & Rothman, 1999).

One of the most abundant of these amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), has been shown to induce breast, colon and prostate tumours in rats (Felton *et al*., 2004). Although HAs are potent mutagens and moderately potent carcinogens at numerous organ sites in rodents and in the liver of non-human primates (Wogan *et al*., 2004), their carcinogenic potential in humans has not been established (Sinha & Rothman, 1999). A positive association between prostate cancer risk and meat consumption has been described by some epidemiologic studies, but underlying mechanisms have not been identified (Norrish *et al*., 1999). Well-done meat has been associated with increased risks of colorectal and breast cancers in humans (Norrish *et al*., 1999). Human dietary epidemiology studies suggest a strong correlation between either meat consumption or well-done muscle meat consumption and cancers of the colon, breast, stomach, lungs and esophagus (Felton *et al*., 2004).

Endogenous risk factors in cancer

The importance of endogenous oxidative damage in carcinogenesis has been recognised (Ames *et al*., 1995). Survival of aerobic organisms in an oxygen environment requires complex defense and repair processes responsible for balancing formation and control of reactive oxygen species (ROS).

Reactive oxygen species (ROS)

Free radicals are chemical species that contain one or more unpaired electrons (Halliwell *et al*., 1992) and are implicated in several degenerative conditions and diseases in humans (Comporti, 1985; Namiki, 1990; Ames & Gold, 1991; Halliwell, 1994; Martínez-Cayuela, 1995; Sastre *et al*., 2000; Kawanishi *et al*., 2001). Biologists use the terms reactive oxygen species (ROS) and oxygen-derived species to include oxygen radicals $O_2^{\bullet-}$, $OH^{\bullet}$, $LOO^{\bullet}$ and $LO^{\bullet}$ as well as nonradical oxygen-containing reactive species HOCL, H_2O_2, O_2 and O_3 (Halliwell *et al*., 1995).

The superoxide anion radical ($O_2^{\bullet-}$) is relatively unreactive, but can be converted to H_2O_2 by superoxide dismutase to take part in the Fenton reaction catalysed by transition metals (copper or iron) (Namiki, 1990). This results in the formation of the very reactive hydroxyl radical ($OH^{\bullet}$) (Namiki, 1990; Collins, 1999). Alternatively the superoxide anion radical ($O_2^{\bullet-}$) can react in the Haber-Weiss reaction (Namiki, 1990).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad \text{(Fenton reaction)}$$

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + O_2^{\bullet-} \rightarrow OH^{\bullet} + OH^- + O_2 \quad \text{(Haber-Weiss reaction)}$$

The Haber-Weiss reaction is not considered to be significant in biological systems, since it is a very slow reaction (Namiki, 1990), but it can lead to the formation of the $OH^{\bullet}$ radical in the presence of iron. The $OH^{\bullet}$ radical is very reactive and reacts oxidatively with all biological materials by hydrogen withdrawal, double bond addition, electron transfer, radical formation, initiation of autoxidation, polymerisation and fragmentation (Namiki, 1990).

Exposure of living cells to potentially damaging free radical species occur mainly through two routes. Intracellular generation as a product of normal cellular metabolism during electron transfer processes mediated by transition metals like iron and copper or by enzymes, or as a consequence of exposure to ultraviolet, visible, thermal or ionizing radiation (Dargel, 1992; Evans *et al*., 2004). ROS play different roles *in vivo* and are generated deliberately when employed for metabolism of foreign matter, antibacterial cell activities (Namiki, 1990) and to carry out certain biological reactions (Martínez-Cayuela, 1995), or accidentally through leakage of electrons onto O_2 from mitochondrial electron transport chains, microsomal cytochrome P450 and electron-donating enzymes, and other systems (Halliwell *et al*., 1995). Potential cellular sources of free radicals are summarised in Table 1.

The metabolism of a number of xenobiotics also results in the production of free radicals (Comporti, 1985; Collins, 1999). Adverse effects of ROS include contribution to cellular ageing (Sastre *et al*., 2000), mutagenesis (Takabe *et al*., 2001), carcinogenesis (Kawanishi *et al*., 2001), and coronary heart disease (Khan & Baseer, 2000), which occur due to destabilisation of membranes (Mora *et al*., 1990), DNA damage (Takabe *et al*., 2001) and oxidation of low-density lipoprotein (LDL). The major cellular and extracellular targets for reactive radical species are proteins, unsaturated fatty acyl components of lipids and lipoproteins and DNA constituents (Rice-Evans & Bruckdorfer, 1992). For the purpose of this study, only oxidative DNA damage and lipid peroxidation will be discussed.

Endogenous and exogenous antioxidants, such as obtained from the diet, are important in humans as protection against free radical damage (Pietta, 2000). Defense systems do not simply consist of scavenging of aggressive intermediates, but include prevention of free radical formation, inhibition of propagation, and repair of lesions (Dargel, 1992). Endogenous defenses against free radical damage *in vivo* include superoxide dismutase (SOD), that converts superoxide to hydrogen peroxide, and glutathione peroxidase, a major enzyme that removes hydrogen peroxide generated by SOD (Halliwell, 1994). Antioxidant defenses are, however, not completely efficient and increased free radical formation in the body will enhance cell damage, which is often referred to as oxidative stress.

Oxidative DNA damage

Sequence-specific DNA damage can be caused by reactive species generated by chemicals and UV radiation and play an important role in mutagenesis, carinogenesis and aging (Kawanishi *et al*., 2001). Free hydroxyl radicals cause DNA damage with no site specificity. The oxidation potentials of DNA bases, however, contribute to determination of sequence specificity of DNA damage, which results in guanine, with the lowest oxidation potential of the four DNA bases, to be most easily oxidised (Kawanishi *et al*., 2001).

$OH^{\bullet}$ radicals are suggested to be the main source of oxidative DNA damage (Rice-Evans & Bruckdorfer, 1992) and interact with DNA by direct abstraction of a hydrogen atom from the DNA deoxyribose-phosphate backbone, resulting in DNA cleavage at every nucleotide without a marked site specificity (Celender & Cech, 1991). A variety of oxidative products are also formed as a result of $OH^{\bullet}$ causing addition to the DNA bases (Kawanishi *et al*., 2001), but the main product is 8-oxo-7,8-dihydro-2'-deoxyguanosine (Fig. 2) (Farmer, 2004).

Table 1 Potential cellular sources of free radicals (Dargel, 1992).

Organelles	**Reaction**
Endoplasmic reticulum	Cytochrome P450 dependent electron transport
	Cytochrome b_5 dependent electron transport
Mitochondria	Electron transport (respiratory chain)
	Lipid peroxidation
Lysosomes	Myeloperoxidase system
Membranes	Lipid peroxidation
	Lipoxygenase
	Prostaglandin synthase
	NADPH-oxidase
Peroxisomes	Oxidases
	Flavoproteins
Cytosol	Hemoglobin
	Oxidases
	Transition metals

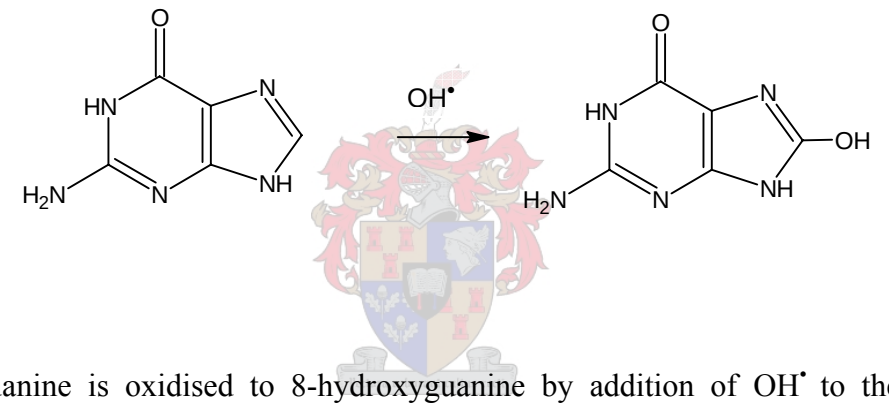


Figure 2 Guanine is oxidised to 8-hydroxyguanine by addition of $OH^{\bullet}$ to the C8 position (Newcomb & Loeb, 1998).

Other oxidation products such as malondialdehyde (a lipid oxidation product) interact with DNA to form promutagenic lesions (Marnett, 1999).

Lipid peroxidation

Auto-oxidation of lipids, known as lipid peroxidation in living systems, may be initiated enzymatically or non-enzymatically (Ratty & Das, 1988) and is caused by species such as $OH^{\bullet}$, alkoxyl radicals $RO^{\bullet}$, peroxyl radicals $ROO^{\bullet}$ and alkyl radicals $R^{\bullet}$ (Rice-Evans & Bruckdorfer, 1992). Cellular membranes are vulnerable to injury due to the susceptibility of polyunsaturated fatty acid side chains of its phospholipid component to peroxidation (Rice-Evans & Bruckdorfer, 1992). Although most molecules in living systems are non-radicals, new radicals are generated through reaction of radicals with non-radicals (Halliwell *et al*., 1995). The formation of reactive radicals *in vivo* is likely to set off free-radical chain reactions. Lipid peroxidation can be set in motion when conditions of increased oxidative stress occur in the cell through increased production of free radicals and/or decreased antioxidant defenses (Comporti, 1985).

Autoxidation, which is a free radical chain process, involves different steps, namely initiation, propagation and termination (Porter *et al*., 1995; Montine *et al.,* 2002). Any primary free radical with sufficient reactivity to extract a hydrogen atom from a reactive methylene group from unsaturated fatty acids can be responsible for the initiation of lipid peroxidation (Rice-Evans & Bruckdorfer, 1992).

Initiation: $In^{\bullet} + RH \rightarrow InH + R^{\bullet}$

Propagation: $R^{\bullet} + O_2 \xrightarrow{k_p} ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow R^{\bullet} + ROOH$

Termination: $2ROO^{\bullet} \rightarrow [ROOOOR]$

Initiation involves the formation of a lipid radical, $R^{\bullet}$ (Sevanian & Ursini, 2000), which occurs through thermal or photochemical hemolytic cleavage of an RH bond or by hydrogen atom abstraction from RH by an initiator free radical (Fig. 3) (Porter *et al*., 1995). The following step, propagation, consists of two steps of which the first involves the addition of molecular oxygen to $R^{\bullet}$. In the second, rate limiting, step of propagation a hydrogen atom is abstracted from RH by

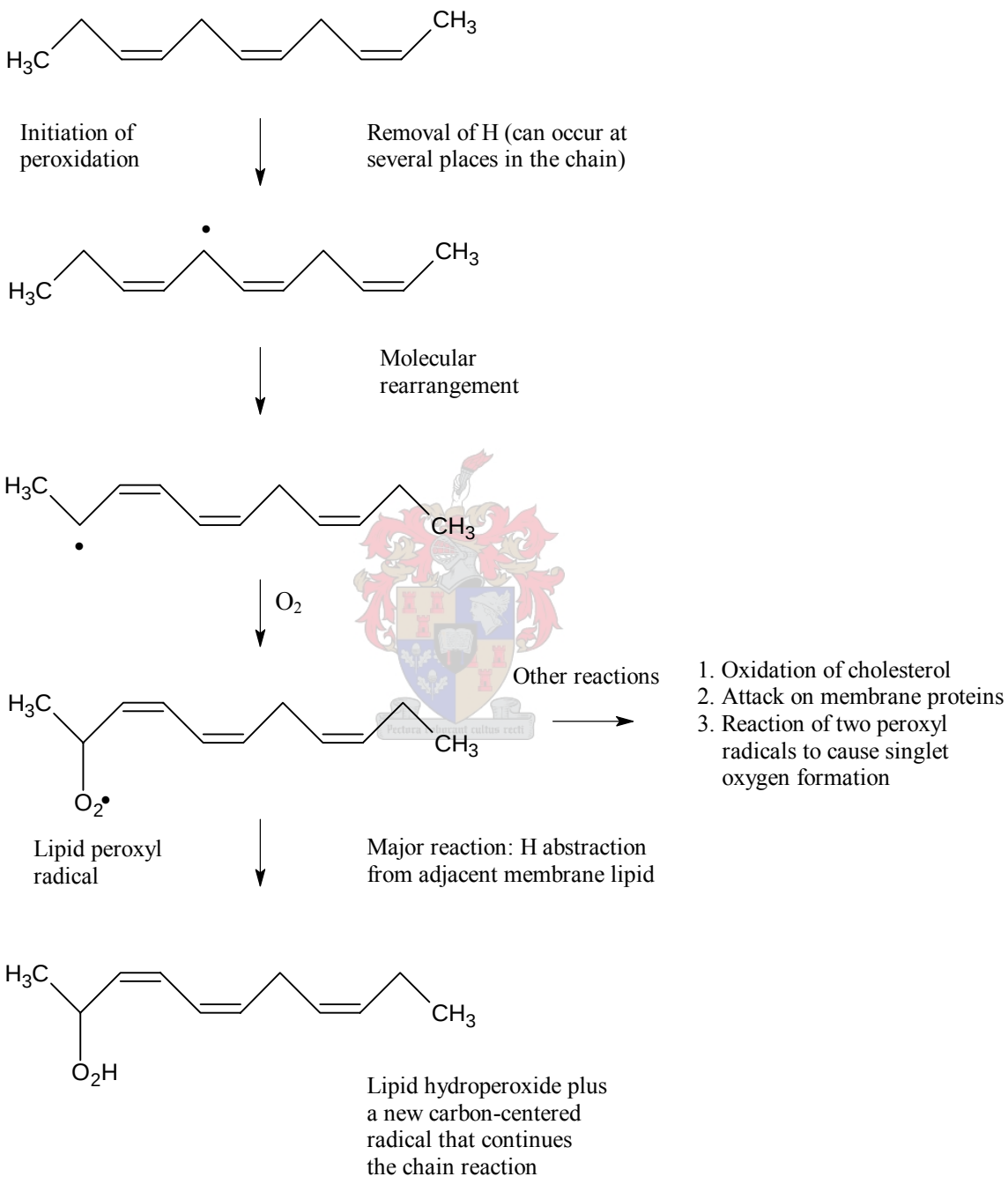


Figure 3 Mechanisms involved in lipid peroxidation (Halliwell *et al*., 1995).

the peroxyl radical ROO$^{\bullet}$ which results in the generation of ROOH and another radical R$^{\bullet}$ (Porter *et al*., 1995). The rate constant k_p depends on the strength of the C-H bond being broken. The chain reaction is terminated by reactions between radicals producing dimers and higher polymers (Wheatley, 2000).

Aldehydes are produced when lipid hydroperoxides break down in biological systems (Esterbauer & Cheeseman, 1990). Identification of these aldehydes is of interest, since it can serve as an index of the extent of lipid peroxidation and as an aid to elucidate the role of aldehydes in certain pathological conditions. It has been postulated that malondialdehyde (MDA) metabolism occurs in most cells (Draper *et al*., 1986) and measurement of MDA by the thiobarbituric (TBA) method has become common practise as an index of lipid peroxidation in cells and tissues (Diplock *et al*., 1994). This test involves the reaction of one molecule of MDA with two molecules of TBA (Esterbauer & Cheeseman, 1990). The reaction results in the formation of a pink pigment that has an absorption maximum at 532-535 nm.

The validity of MDA as an index of lipid peroxidation is often questioned due to MDA formation as an artifact of analysis and as a product of enzyme reactions, its occurance in various bound forms, and the specificity of methods used for its measurement (Draper & Hadley, 1990). On the other hand, using this method as an indication of lipid peroxidation has also been found to be a reliable indicator and it has been shown that difficulties in MDA determination can be resolved by appropriate modification of methodology. *In vitro* MDA interacts with and alters many biomolecules, including proteins, DNA and RNA (Esterbauer & Cheeseman, 1990).

D. Carcinogen metabolism

Enzyme systems involved in carcinogen metabolism are very complex and apart from cytochrome P450 and gluthathione transferases comprise peroxidases, quinone reductases, UDP-glucuronyltransferases, flavin-containing monooxygenases, epoxide hydrolases, and sulphotransferase (Bailey & Williams, 1994). Although it does not fall within the scope of this study, it is important to note that the activity of any particular enzyme can vary with species, organ cell type, developmental age, genetic predisposition, and recent history of exposure to enzyme inhibitions, inducers, or repressors (Bailey & Williams, 1994).

The potential of a particular foodborne carcinogen to damage DNA in a cell does not merely depend on the carcinogen dose or potency to induce mutations, but also on the balance of enzymes competing for activation and detoxication of that compound in the species, individual,

and target organ of concern. Dietary modulators affect the balance of these competing enzymic pathways by selective enzyme induction or inhibition (Bailey & Williams, 1994).

Types of carcinogens: different mechanisms of action

Carcinogens can mechanistically be divided into genotoxic and non-genotoxic compounds (Bailey & Williams, 1994). Genotoxic carcinogens react with nucleic acids (Reddy *et al*., 2003), have the ability to cause mutations and are DNA reactive in rapid bioassays, while non-genotoxic compounds do not react with DNA, can be viral or chemical and operate by mechanisms such as alterations of endocrine or immune status (Weisburger, 2001). The majority of food-related genotoxins, which include mycotoxins, PAHs, N-nitrosoamines and HA's are not carcinogenic until converted by cellular enzymes into highly electrophilic metabolites capable of interacting covalently with DNA in the target organ (Bailey & Williams, 1994). Carcinogens that require metabolic activation to induce carcinogenesis are also referred to as procarcinogens (Chen & Yen, 1997; Reddy *et al*., 2003).

AFB_1 is a procarcinogen metabolised into several metabolites, which include the main reactive intermediate, aflatoxin 8,9-oxide (Fig. 4) (Yasushi & Kato, 1993). It has been shown in the *Salmonella* mutagenicity assay and the *umu* response tests that several cytochrome P450's mediate the activation of AFB_1 (Yasushi & Kato, 1993).

Cytochrome P450

Cytochrome P450 is the collective name used for a large group of hemoproteins with the same ligand structure around the heme (Omura, 1993). This group of enzymes are heme-containing mixed function oxidases which play a role in metabolism of hydrophobic endogenic substrates (sterols, prostaglandins, fatty acids) and xenobiotics, ingested foreign compounds i.e. drugs, carcinogens, food components and pollutants (Nebert & Dieter, 2000). The metabolism of endogenous and exogenous substrates is catalysed by cytochrome P450, but much more is known about metabolism of the exogenous substrates (Kamataki, 1993). Describing this enzyme as a cytochrome is not precise since it differs significantly from other cytochromes in several ways and it is a heme-thiolate enzyme rather than a redox protein (Lewis, 2001). Cytochrome P450 is present in most tissues in mammalian species, but the greater proportion is found in the liver, located in the smooth endoplasmic reticulum (ER) membrane of hepatocytes (Lewis, 2001).

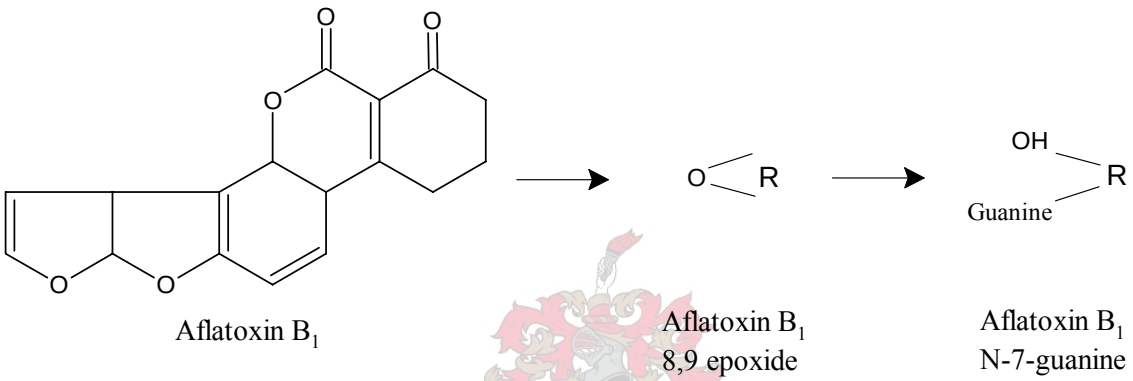


Figure 4 Metabolism of the procarcinogen AFB_1 to the main reactive intermediate, aflatoxin 8,9-oxide, which can interact with DNA (Bailey & Williams, 1994).

Detoxifying and activation of xenobiotics that occurs in liver microsomes has been recognised as drug metabolising enzyme activity and is principally performed by cytochrome P450 (Kamataki, 1993). Many different forms of P450 have been shown to exist in liver microsomes with distinguishable, but overlapping, substrate specificity to foreign substances. Primary metabolism by cytochrome P450 is responsible for production of reactive metabolites, while secondary metabolism (conjugating) is mainly responsible for detoxifying. The detoxifying function of cytochrome P450 includes oxidative metabolism of foreign compounds and drugs to make these hydrophobic foreign compounds more polar as the first step of their excretion from the body (Omura, 1999).

However, oxidation of some chemical compounds by cytochrome P450 catalysed reactions produces highly reactive metabolites that have the ability to react with proteins, and nucleic acids causing cytotoxicity and genotoxicity. The major role of cytochrome P450 in the metabolic activation of various carcinogens was confirmed in the beginning of the 1970's and resulted in a large expansion of research on cytochrome P450 (Omura, 1993).

The catalytic cycle of cytochrome P450-dependent monooxygenase reactions

Cytochrome P450 catalyses reactions in which an organic substrate (RH) combines with oxygen to produce a molecule of water and a mono-oxygenated metabolite (ROH) (Nelson & Cox, 2000) (Fig. 5). The two reducing equivalents are supplied by NADH or NADPH and transferred in two successive stages via one or two redox partners (Lewis, 2001). Redox partners depend on the cytochrome P450 enzyme concerned and may include either an iron-sulphur redoxin, a flavoprotein or cytochrome b_5.

The endoplasmic reticulum (the microsomal fraction) contains membrane bound non-phosphorylating electron-transport systems that take part in hydroxylation and desaturation reactions (Nelson & Cox, 2000). One of the microsomal electron-transport systems of the liver consists of a cytochrome P450 and a flavoprotein, NADPH-cytochrome P450 reductase, which are tightly bound to the microsomal membrane (Takemori *et al*., 1993). Microsomal cytochrome P450 linked electron transport systems differ from the mitochondrial system, since an iron-sulfur protein is not required as the electron carrier between the flavoprotein and cytochrome P450. In the mitochondrial system the reductase contains a single molecule of FAD, while NADPH cytochrome P450 reductase in the endoplasmic reticulum contains one molecule each of FMN and FAD (Takemori *et al*., 1993).

In the microsomal electron transport system FAD serves as the electron acceptor from NADPH and FMN as the eletron transfer intermediate between the reduced FAD and cytochrome P450 (Takemori *et al*., 1993). Cytochrome b_5 is also a possible component of this electron transfer system and responsible for distribution of reducing equivalents.

The scheme presented in Fig. 6 presents the cytochrome P450 catalytic cycle with the oxidised and the reduced states presented by Fe^{+3} and Fe^{+2}, respectively (Kominami, 1993).

Characterisation of cytochrome P450

P450 was initially characterised by the UV spectra of its reduced carbon monoxide (CO) complex, from where the name cytochrome P450 originated, since it absorbed light strongly at 450 nm (Fig. 7) (Omura & Sato, 1962).

Omura & Sato (1964b) assumed a quantitative conversion of P450 to P420 and calculated an extinction coefficient value for the CO difference spectrum of reduced cytochrome P450. This value was calculated as 91 cm^{-1} mM^{-1} for the absorbance difference between 450 nm and 490 nm, which enabled the calculation of the P450 content in microsomes.

Hepatic cytochrome P450 substrate binding

Detection and characterisation of cytochrome P450 have involved extensive use of UV/visible spectrophotometry, since substrate binding has a distinct effect on the appearance of the overall spectrum, particularly with respect to the major absorption bands (Omura & Sato, 1962; Omura & Sato, 1964a; Scenkman *et al*., 1967; Schenkman, 1970; Schenkman *et al*., 1981).

Characteristics in cytochrome P450 electronic spectra are due to energy level transition within the hemelocus of the enzyme itself, which are influenced by the nature of the heme ligands, the environment of the heme moiety and the presence of a bound substrate or inhibitor (Lewis, 2001). Three specific types of substrate binding difference spectra have been identified and are referred to as Type I, Type II and Reverse Type I (also referred to as Modified Type II) (Schenkman, 1970; Schenkman *et al*., 1981). When a maximum absorption in the difference spectra is observed at 385-390 nm and a minimum at about 420 it is termed a **Type I** spectral change (Remmer *et al*., 1966; Schenkman *et al*., 1967). Aminopyrine, phenobarbital and hexobarbital are only a few of the substrates that induce this type of spectral change. Type I binding is associated with heme ligation of cytochrome P450 (Schenkman *et al.* 1981). Another class of spectral change, **Type II,** is characterised by the appearance of an absorption peak at approximately 430 nm and a minimum at 390 (Remmer *et al.,* 1966; Schenkman *et al*., 1967).

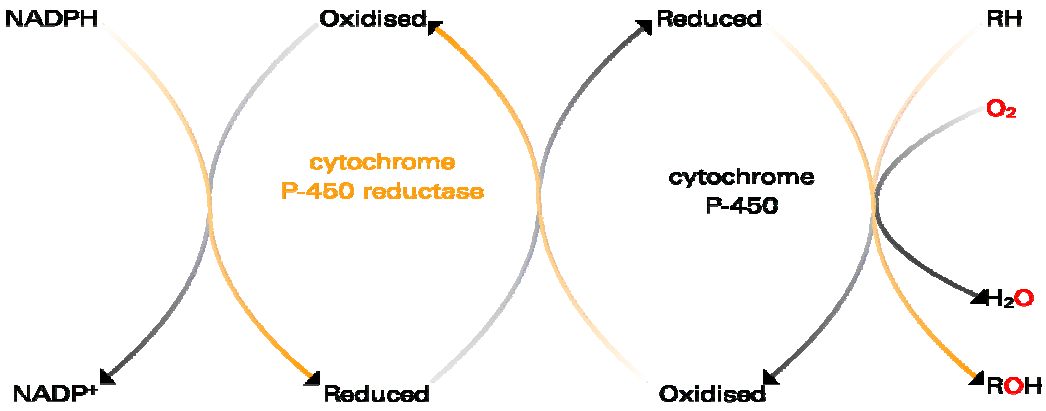


Figure 5 A simplified diagram showing cytochrome P450 mediated hydroxylation of a substrate, which has intermediate steps that is not yet fully understood (Nelson & Cox, 2000).

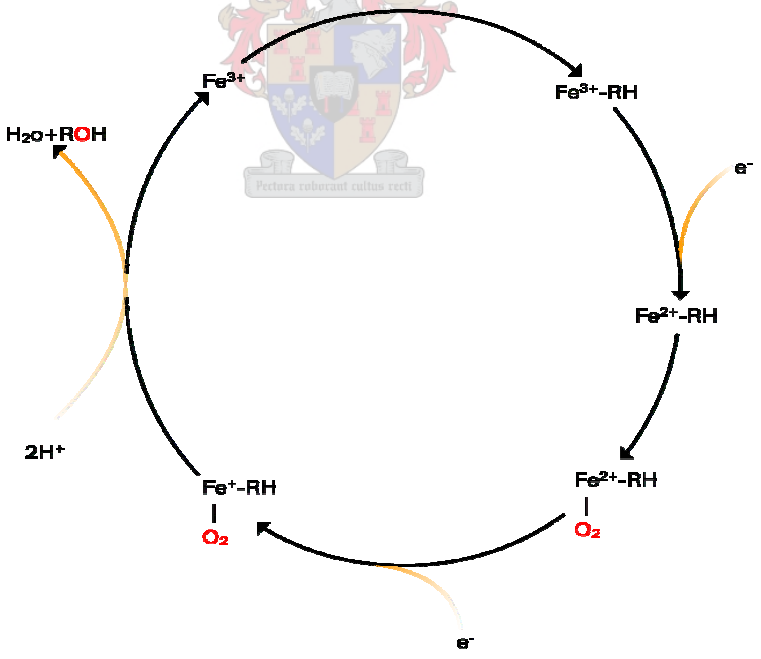


Figure 6 The reaction cycle of cytochrome P450 reactions with the stages of the substrate becoming oxygenated and the cytochrome P450 heme iron redox state at different stages of the reaction (Lewis, 2001).

This type of spectral change is the result of addition of substrates such as aniline and pyridine to liver microsomes. The **Reverse Type I (RI)** spectral change is characterised by maximum absorption at 420 nm and a minimum at about 392 nm in the difference spectra (Schenkman *et al*., 1972). The Reverse Type I change is characteristically a "mirror-image" of the Type I and was originally referred to as Modified Type II because of its resemblance to the Type II binding spectrum (Lewis, 2001). This binding type does however, not only differ from the Type II binding in terms of the minimum and maximum absorbance, but it is thought to be the result of displacement of the distal ligand (water molecule or hydroxide ion) combined with substrate binding to the hydrophobic region of the heme pocket, instead of heme ligation (Type II) (Schenkman *et al*., 1981).

The Type II spectral change (Fig. 8) is similar to the reaction between a basic amine and a ferrihemoprotein (Schenkman *et al*., 1967). It has been shown that the spectral change induced by Type II substrates is due to the formation of a ferrihemochrome (Schenkman, 1970; Schenkman *et al*., 1973). The maximum absorbance varies between 425 nm and 435 nm depending upon which amine is added. It has been shown that aniline, a Type II substrate, can displace carbon monoxide from the reduced hemoprotein, which indicates interaction with the heme iron. Aniline has also been shown to react with reduced cytochrome P450 to form a spectral change containing two Soret peaks (Imai & Sato, 1967). The magnitude of these spectral changes is proportional to the content of P450 (Imai & Sato, 1967) and the protein concentration (Remmer *et al*., 1966; Schenkman *et al*., 1967) of the liver microsome preparation. The concentration of the substrate (Fig. 8) added to the microsomal suspension also influences the magnitude of the spectral change (Schenkman, 1970).

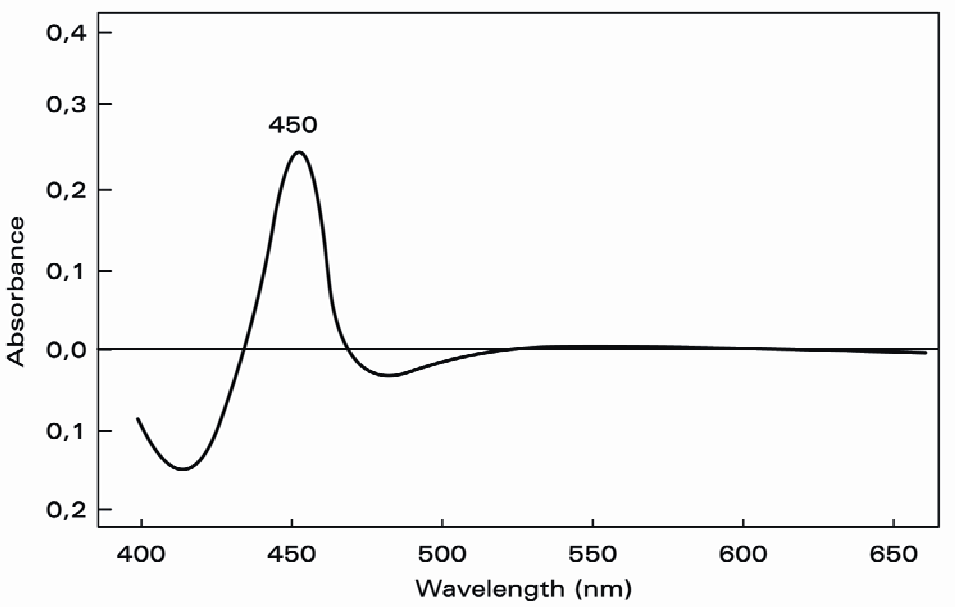


Figure 7 Carbon monoxide difference spectra of liver microsomes reduced with a few milligrams of $Na_2S_2O_4$ and saturated with CO (Omura & Sato, 1962).

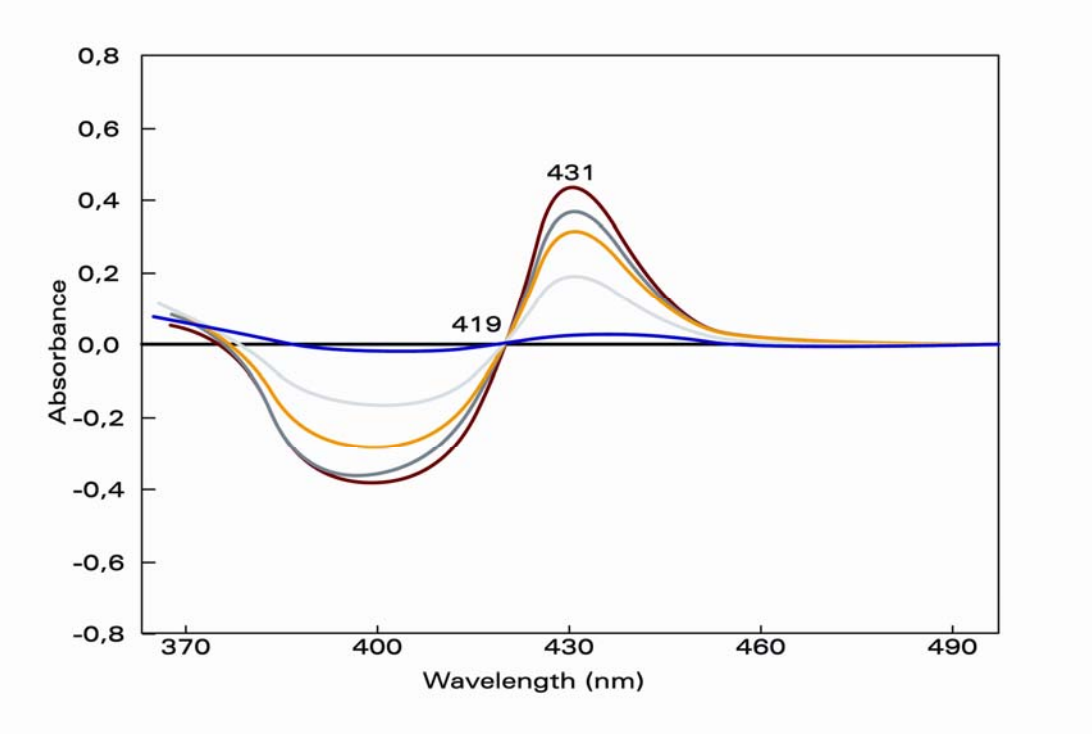


Figure 8 Difference spectra of aniline induced Type II spectral change with liver microsomes, using the following different aniline concentrations — 0.037 mM, — 0.224 mM, — 0.690 mM, — 1.620 mM, — 3.700 mM (Schenkman, 1970).

E. Cancer chemoprevention

Cancer chemoprevention developed as a promising strategy for prevention together with progress in an understanding at cellular and molecular levels of carcinogenesis (Surh, 1999). This strategy refers to administration of natural or synthetic compounds which are non-toxic, to inhibit or reverse the process of carcinogenesis (Tanaka, 1997; Surh, 1999). Micheal Sporn who used retinoids to stop the progress of experimental carcinogenesis used the term chemoprevention for the first time (Sporn & Roberts, 1984). Daily exposure to environmental carcinogens from diverse sources renders ways to neutralise such carcinogens or protect against their deleterious effects essential (Surh, 1999).

Classification of chemopreventive agents and mechanisms of prevention

Wattenberg (1985) proposed the first conventional classification system of chemopreventive agents. This classification was based on the underlying mechanisms through which an agent protects in a specific stage of the multistage cancer process. In this system chemopreventive agents are divided into two major categories, blocking agents and suppressing agents. The hypothetical sites where the two types of anticarcinogens interfere with the carcinogenesis process is shown in Fig. 9.

Blocking agents are compounds that inhibit initiation either by inhibiting the formation of carcinogens from precursor molecules or reactive metabolites from the parent carcinogens. Compounds that prevent interaction of the ultimate ellectrophilic and carcinogenic species with cellular target molecules, such as DNA, RNA and proteins, are also classified as blocking agents. According to this scheme the second major category, **suppressing agents**, are responsible for inhibition of malignant expression of initiated cells, in either the promotion or the progression stage (Wattenberg, 1985).

Kada & Shimoi (1987) additionally proposed two similar ways of preventing induced cellular mutagenesis, with different cataloging. In the first, antimutagens are responsible for inactivating mutagens before they attack DNA and are referred to as **desmutagens**.

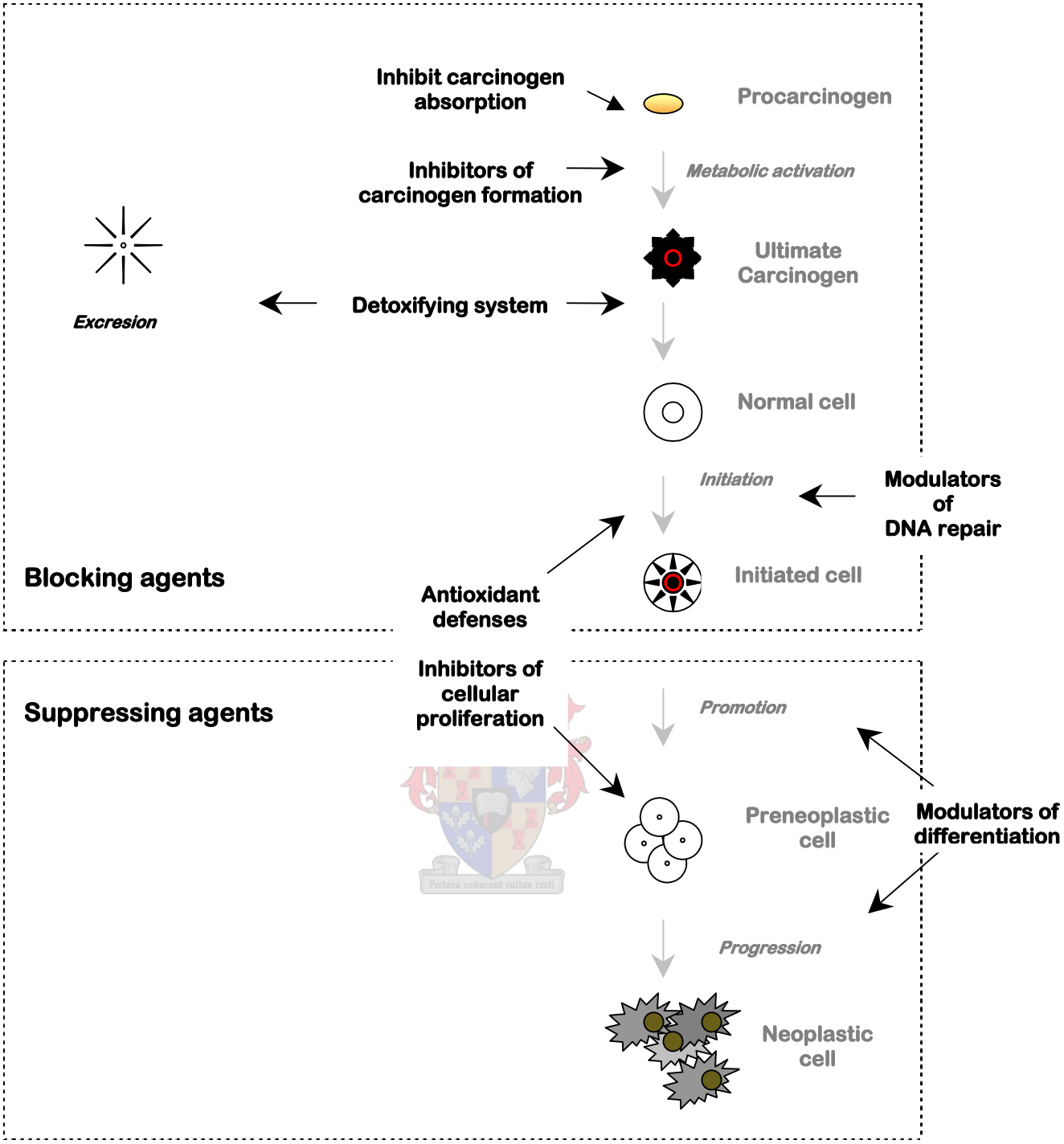


Figure 9 Hypothetical cites of interaction of dietary anticarcinogens with the multi-step carcinogenesis process. Blocking agents are the anticarcinogens responsible for prevention of initiation, while suppressing agents inhibit development of tumours from the initiated cell (modified from Johnson, 2003).

The second strategy involves **bio-antimutagens**, which interfere with cellular fixation processes of DNA damage.

In contrast to the above classification systems it has been shown that multiple genetic changes occur along all stages of carcinogenesis (Fearon & Volgstein, 1990) and that the development of cancer should rather be viewed as a continuum of mutagenic and mitogenic events, which does not fit a rigid scheme of multiple, separate phases (Boone *et al*., 1992). Therefore classification of cancer chemopreventive agents should distinguish inhibitors based on their intervention level throughout the cancer process. De Flora & Ramel (1988) stated that the sequence of events occurring during the carcinogenesis process should not be oversimplified by rigid classification into definite steps involved in the initiation-promotion-progression operative scheme. This is taken into account in the detailed classification of chemopreventive agents by De Flora (1998) presented in Table 2. The classification system presented in Table 2 presents analysis of extracellular and intercellular inhibition of mutations and cancer initiation, followed by mechanisms interfering with the later stages of carcinogenesis. Mechanisms, for instance inhibition of genotoxic effects, antioxidant activity and scavenging of free radicals, induction of apoptosis and signal transduction modulation, are repeated in the system as it occurs in different phases of the process. Some of the mechanisms are also strictly interconnected and partially overlap.

Natural dietary constituents as antimutagens and anticarcinogens

Considerable attention has lately been focused on identifying **natural** occurring chemopreventive substances present in plants (Surh, 1999; Kohlmeier *et al*., 1997). Reduced risk of cancer and cardiovascular disease associated with intake of fruit and vegetables has primarily been attributed to beta-carotene and ascorbate, while phenolic constituents may also play a role (Heim *et al*., 2002).

Several phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess significant anticarcinogenic and antimutagenic activities, which is thought to be due in part to their antioxidative and anti-inflammatory properties (Surh, 1999).

Of the plant phenolics, flavonoids are of particular interest due to their wide distribution in dietary plants and frequent consumption with dietary intake estimates in Western countries varying from 23 mg to 1000 mg per day (Hertog *et al*., 1993; Peterson & Dwyer, 1998).

Table 2. Classification of mechanisms of inhibitors of mutagenesis and carcinogenesis (adapted from De Flora, 1998).

1. Extracellular mechanisms
   a) Inhibition of uptake of mutagens/carcinogens
      - Inhibition of penetration
      - Removal from the organism
   b) Inhibition of the endogenous formation of mutagens and carcinogens
      - Inhibition of nitrosation reaction
      - Modification of the intestinal microbial flora
   c) Complexation, dilution and/or deactivation of mutagens/carcinogens
      - Physical or mechanical means
      - Chemical reaction
      - Enzyme-catalysed reaction
   d) Favouring absorption of protective agents
2. Inhibition of mutation and cancer initiation by cellular mechanisms
   a) Stimulation of trapping and detoxification in non-target cells
   b) Modification of transmembrane transport
      - Inhibition of cellular uptake
      - Stimulation of extrusion outside cells
   c) Modulation of metabolism
      - Inhibition of activation of promutagens/procarcinogens by phase I enzymes
      - Induction of phase I detoxification and phase II conjugation pathways, or acceleration of decomposition of reactive metabolites
      - Stimulation of activation, coordinated with detoxification and blocking of reactive metabolites
   d) Blocking or competition
      - Trapping of electrophiles by either chemical reaction or enzyme-catalysed conjugation
      - Protection of DNA nucleophilic sites
      - Protection of DNA
   e) Inhibition of cell replication
   f) Modulation of DNA metabolism and repair
      - Increase of fidelity of DNA replication and repair
      - Stimulation of repair and/or reversion of DNA damage
      - Inhibition of error-prone repair pathways
      - Correction of hypomethylation
   g) Control of gene expression
      - Inhibition of oncogene expression
      - Inhibition of oncogene sequences
      - Inhibition of translation targeted to oncogene mRNA
      - Inhibition of transcription of specific DNA sequences
      - Site-specific DNA binding

Neutralisation of oncogene products

Replacement of deleted tumour suppressor genes

Killing of cells lacking tumour suppressor genes

3. Inhibition of tumour promotion
    a) Inhibition of genotoxic effects
    b) Antioxidant activity and scavenging of free radicals
    c) Inhibition of proteases
    d) Inhibition of cell proliferation
    e) Induction of cell differentiation
    f) Induction of cell apoptosis
    g) Protection of intercellular communications
    h) Signal transduction modulation
4. Inhibition of tumour progression
    a) Inhibition of genotoxic effects
    b) Antioxidant activity and scavenging of free radicals
    c) Inhibition of proteases
    d) Signal transduction modulation
    e) Effects on the hormonal status
    f) Effects on the immune system
    g) Inhibition of neovascularisation
    h) Physical, chemical, or biological antineoplastic activity
5. Inhibition of invasion and metastasis
    a) Inhibition of proteases involved in basement membrane degradation and modulation of the interaction with the extracellular matrix
    b) Induction of cell differentiation
    c) Inhibition of neovascularisation
    d) Effect on cell-adhesion molecules
    e) Antioxidant activity
    f) Signal transduction modulation
    g) Activation of animetastasis genes

Flavonoids

Flavonoids are a broad class of secondary plant phenolics found widely distributed in leaves, seeds, bark and flowers of plants, offering protection against ultraviolet radiation, pathogens and herbivores (Harborne, 2000). These phenolics are also important constituents of the human diet, are found in fruits, vegetables, seeds, as well as tea and wine (Harborne, 2000), and have been extensively studied for their role in human health (Siess *et al*., 2000). Flavonoids have existed in nature for over one billion years and it is thought that this long association with various animal species and other organisms throughout evolution may account for the extraordinary range of biochemical and pharmacological activities in mammalian and other cell systems (Middleton & Kandaswami, 1993).

Physiological and pharmacological properties of flavonoids

Polyphenols contribute significantly to the taste and flavour of food, and products containing large quantities are generally described as *astringent* (Haslam, 1989). Beneficial health effects of flavonoids are mainly attributed to their antioxidant and metal chelating abilities (Heim *et al*., 2002; Middleton & Kandaswami, 1993), as well as the ability to modulate the activity of several enzymes or cell receptors (Hodek *et al*., 2002). Incomplete efficiency of endogenous defense systems and exposure to factors such as cigarette smoke, air pollutants, UV radiation, high polyunsaturated fatty acid diet and inflammation that increase ROS production, make the intake of dietary antioxidants essential to diminish effects of oxidative damage over the human life span (Hallliwell, 1994). Beneficial biological activities of flavonoids that have been recognised include anti-bacterial and anti-viral activity, anti-inflammatory, anti-angionic, analgesic, anti-allergic effects, hepato-protective, cytostatic, apoptotic, antimutagenic/anticancer, estrogenic and anti-estrogenic properties (Heim *et al*., 2002).

Dietary constituents with antioxidant and/or free radical scavenging activity sometimes show antimutagenic and/or anticarcinogenic properties, as a number of mutagens and carcinogens act through generation of various oxygen radicals, which are genotoxic and may lead to generation of other radicals (Wang *et al*., 1989). This antimutagenic activity is attributed to protection against *in vivo* generation of genotoxic intermediates. In addition to radical scavenging, flavonoids exert a highly specific effect on crucial regulatory enzymes and receptors in organisms (Heim *et al*., 2002). Flavonoids have demonstrated cardioprotective effects by virtue of their capacity to inhibit LDL oxidation (Kondo *et al.,* 1996; Mazur *et al*., 1999).

The chemical structure and some activities of several flavonoids are similar to those of naturally occurring estrogens and they are therefore frequently assigned as phytoestrogens (Das *et al*., 1994; Hodek *et al*., 2002). It is important to note that not all flavonoids have beneficial effects, since it has been reported that some are mutagenic and/or prooxidants and interfere with essential biochemical pathways (Hodek *et al*., 2002).

Prooxidant activity and mutagenicity related to flavonoid mediated oxidative damage

Reports of mutagenicity related to flavonoid mediated oxidative damage (Sugimura *et al*., 1977; Elliger *et al*., 1984; Beudot *et al*., 1998; Yamashita, 1999; Yoshino *et al*., 1999) raised obvious concerns, since concentrated extracts of flavonoid-rich plants such as propolis, pine bark, green tea leaves, soy isoflavones and grape seed are widely marketed as nutraceuticals. These products are specifically aimed at slowing down ageing and at individuals with conditions such as cardiovascular disease, cancer and chronic inflammation.

Mutagenicity of several naturally occurring flavonoids have been studied in the *Salmonella typhimurium* mutagenicity assay, with different strains of bacteria (Das *et al*., 1994). Quercetin and some closely related flavonoids have been reported to induce specific locus mutations, chromosomal aberrations, sister chromatid exchanges, DNA strand breaks and weak cellular transformations (Elliger *et al*., 1984; Sugimura *et al*., 1977; Beudot *et al*., 1998). Despite *in vitro* genotoxicity, carcinogenicity studies of flavonoids in mammals are predominantly negative (Middleton & Kandaswami, 1993; Das *et al*., 1994).

Chemical structure

Plant phenolics are characterised by low molecular weight and the flavan nucleus (Harborne, 2000) and more than 4000 compounds of the flavonoid structure have been identified (Peterson & Dwyer, 1998). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Fig. 10) and are classified according to substitutions. Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups and in the conjugation between the A- and B- rings (Heim *et al*., 2002), with the main dietary flavonoids comprising flavanols, flavones, flavonols, flavanones, isoflavones and anthocyanidins (Table 3) (Heim *et al*., 2002). Polymerization of the flavonoids, which primarily exist as 3-*O*-glycosides and polymers in food, yields tannins and other complex species occurring in red wine, grapes and black tea (Hammerstone *et al*., 2000).

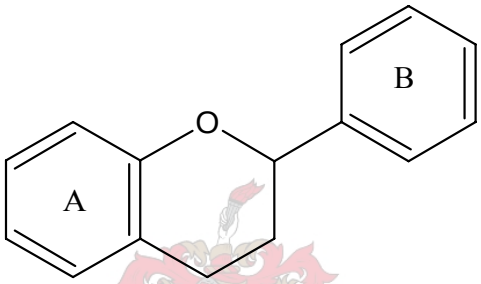
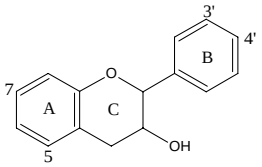
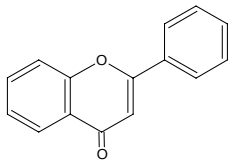
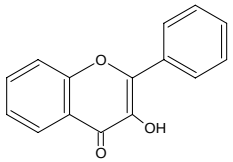
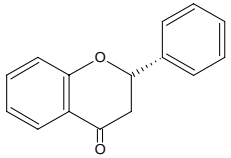
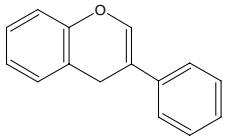
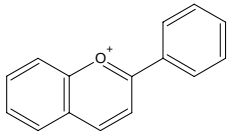


Figure 10 The nuclear structure of flavonoids (Heim *et al*., 2002).

Table 3 Basic structures of dietary flavonoids, substitution patterns and their dietary sources (Heim *et al*., 2002).

Class	General structure	Flavonoid	Substitution pattern	Dietary source
Flavanol		(+) catechin	5,7,3’,4’-OH	tea (*Camellia sinensis*)
		(-) epicatechin	5,7,3’,4’-OH	tea
		epigallocatechin gallate	5,7,3’,4’,5’-OH, 3-gallate	tea
Flavone		chrysin	5,7-OH	fruit skins
		apigenin	5,7,4’-OH	parsley, celery
		rutin	5,7,’3,4’-OH,3-rutinose	red wine, buckwheat, citrus
		luteolin	5,7,3’,4’-OH	red pepper
		luteolin glucoside	5,4’-OH,4’,7-glucose	
Flavonol		kaempferol	5,7,4’-OH	leek, broccoli, endives, grapefruit, black tea
		quercetin	5,7,3’,4’-OH	onion, lettuce, broccoli, tomato, tea, red wine, berries, olive oil
		myricetin	5,7,3’,4’,5’-OH	cranberry grapes, red wine
		tamatixetin	5,7,3’-OH, 4’-OMe	
Flavanone (dihydroflavone)		naringin	5,4’-OH,7-rhamnoglucose	citrus, grapefruit
		naringenin	5,7,4’-OH	citrus fruits
		taxifolin	3,5,7,3’,4’-OH	citrus fruits
		eriodictyol	5,7,3’,4’-OH	lemons
		hesperidin	3,5,3’-OH,4’-OME,7-rutinose	oranges
Isoflavone		genistin	5,4’-OH, 7-glucose	soybean
		genistein	5,7,4’-OH	soybean
		daidzin	4’-OH,7-glucose	soybean
		daidzein	7,4’-OH	soybean
Anthocyanidin		apigenidin	5,7,4’-OH	coloured fruits
		cyanidin	3,5,7,4’-OH,3,5-OMe	cherry, raspberry, strawberry

F. Tea

Rooibos (*Aspalathus linearis*) and honeybush tea (*Cyclopia spp.*) are two popular health drinks indigenous to South Africa. The genus *Cyclopia* has been utilised for its medicinal properties for centuries (Watt & Breyer-Brandwijk, 1932), but has also been enjoyed as a sweet herbal infusion known as honeybush tea, produced from leaves, stems and flowers of *Cyclopia* species (Du Toit *et al.*,1999). Only four of the *ca.* 24 species are currently of commercial importance and include *C. intermedia* E. Mey., *C. subternata* Vogel ,*C. genistoides* (L.) R.Br. and *C. sessiliflora* Eckl. & Zeyh (Du Toit & Joubert, 1998), while small quantities of *C. maculata* is also harvested and processed (Dr. E Joubert, ARC Infruitec-Neitvoorbij, Stellenbosch, personal communication, 2004). Rooibos tea has received much attention and increased popularity due to beneficial effects (Snyman, 2000) mostly based on anecdotal evidence. Improvement of appetite, allergies and nervous complaints and the ability to cure insomnia are associated with regular consumption of rooibos tea (Blommaert & Steenkamp, 1978).

There has been dramatic growth in the use of honeybush tea in the past five years and export of this beverage has become a major industry, following on the success of rooibos tea (De Villiers, 2004). Harvesting of honeybush tea has shown significant growth in recent years, from approximately 30 tons processed in 1997 (De Villiers, 2002) to likely exceeding 300 tons in 2004 (De Villiers, 2004). This increase mainly reflects the development of the international market, but substantial growth in domestic consumption is also evident. The tea has been exported to Japan, Germany and Switzerland since 1993 (Du Toit *et al*., 1999)

Growth in the market for rooibos tea is partly attributed to significant development in sophisticated and health conscious markets (Snyman, 2000). The rooibos tea market has expanded from a modest 8.5 tons exported in 1966 to more than 4500 tons in 2000 and foreign earnings of an estimated R20 million in 1999 (Snyman, 2000). Cape Natural Products reported more than 50% growth in export of rooibos tea to Germany and Japan for the period 2001-2003 (Dr. E Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, personal communication, 2004). In 2003 rooibos tea sales totaled 10 600 tons of which 6400 tons was exported and 4200 tons consumed locally. The quantity of tea exported thus exceeded that of domestic use by more than 2000 tons, with more than half of the export sales accounted for by Germany. Additional principle importers of rooibos comprise Japan, the Netherlands and to a lesser extend the United Kingdom and United States of America (Dr. E Joubert, ARC Infruitec-Neitvoorbij, Stellenbosch, personal communications, 2004).

The fragrant brew, tea, that was discovered by a Chinese emperor more than 2000 years ago, is a global drink today enjoyed hot or iced and appreciated for its ability to revive, refresh and relax the body (Balentine & Paetau-Robinson, 2000). The plant and its infusion that was originally utilised as medicine is now cultivated in more than 20 countries (Chu, 1997). The tea plant is taxonomically classified as *Camellia sinensis* (L.) O. Kuntze of the family *Theaceae* and is distinguished from other wild species by its characteristic taste attributed to its content of caffeine, polyphenols and theanine (Chu, 1997). The processing of *Camellia sinensis* in different parts of the world gives rise to green (20%), black (78%) or oolong tea (2%) (Kuroda & Hara, 1999). The leading producers and consumers of tea produced from the *Camellia sinensis* plant are India and China, with other major markets for black tea consumption including Pakistan, Iran and Egypt (Snyman, 2000). The United Nations Food and Agricultural Organisation (FAO) projects a 3% increase in world black tea consumption, to more than 3.2 million tons by 2005.

Processing of tea

The manufacture of honeybush tea in South Africa is still based on traditional methods, and involves cutting the foliage and fine stems into small pieces before fermentation (Du Toit & Joubert, 1999). Heap fermentation has, however, been replaced with high temperature "fermentation" (oxidation) under controlled conditions followed by drying (Dr. E Joubert, ARC, Stellenbosch, personal communication, 2004). Processing of rooibos tea involves cutting, bruising, "fermentation" and drying, which takes place in open air under controlled conditions (Joubert & De Villiers, 1997). Fermentation refers to the chemical oxidation step used in processing for development of the characteristic red to brown colour and is essential for the development of the desired sensory properties such as the sweet flavour of these beverages (Du Toit & Joubert, 1998). Processed tea is often referred to as fermented, while unfermented tea refers to processed tea (crushed and dried) where processing did not involve chemical oxidation (fermentation).

Two varieties of *Camellia sinensis* are used for green and black tea production, with var. *sinensis* generally used for the production of green tea in Japan and China and var. *asamica* to make black tea (Takeo, 1992). Processing of green tea, which is mainly consumed in Japan, China, North America and the Middle East, is designed to prevent enzymatic oxidation of catechins, by inactivating enzymes present in the tea leaves (Balentine, 1996). The manufacturing process of black tea comprises instrumental disruption of the tea shoots to facilitate the mixing of polyphenols held in the cell vacuole with cytoplasmic polyphenol oxidase (Robertson, 1992).

The biochemical and chemical reactions that occur during fermentation for the production of black tea is very complex and only partially understood (Robertson, 1992). It involves the uptake of atmospheric oxygen and the formation of the pigmented, hot water-soluble, polyphenolic compounds, characteristic of black teas.

The enzyme, polyphenol oxidase, is responsible for the promotion of oxidation of catechins to yield theaflavins and thearubigins (Balentine *et al.*, 1997), that provide visual brightness and depth of colour and the characteristic strength and mouthfeel of black tea (Robertson, 1992). Cultivars that show better fermentability and higher content of polyphenols are better for black tea manufacture (Chu, 1997). Most of the cultivars that belong to the group *Camellia sinensis var sinensis* have low fermentability and contain less polyphenols. Fermentability of tea leaves is genetically regulated by the activity of polyphenol oxidase, controlled by a polygene system, and is taken as one of the main marks in the breeding of tea, especially of black tea (Chu, 1997).

Phenolic composition of tea

The phenolic composition of rooibos and honeybush tea is very different from that of the teas produced from *Camellia sinensis*. In contrast with *Camellia sinensis* teas these two herbal teas do not contain caffeine (Greenish, 1881; Blommaert & Steenkamp,1978) and have a low tannin content (Blommaert & Steenkamp,1978; Terblanche, 1982). The phenolic composition of the two types of tea also differ significantly from each other.

Honeybush tea (*Cyclopia spp.)*

Reports of chemical analyses of *Cyclopia* leaves date back to 1870 and 1881 (Greenish, 1881; Watt & Breyer-Brandwijk, 1932) wherein the presence of unknown and unidentified substances were mentioned. De Nysschen *et al.* (1996) screened methanol extracts from the leaves of 22 unprocessed *Cyclopia spp.* for the presence of phenolic compounds and reported three major constituents, a xanthone *C*-glycoside, mangiferin and the two flavanones hesperetin and isosakuranetin.

In support of the establishment of the honeybush tea industry, the phenolic composition of the main natural resource used in production, *C. intermedia*, was reinvestigated (Ferreira *et al*., 1998). The complex mixture of phenolic compounds in methanol extracts of fermented *C. intermedia* consists of the hydroxycinnamic acid, *p*–coumaric acid, a variety of $C_6.C_3.C_6$-type polyphenols and two xanthone *C*–glycosides. Five isoflavones, formononetin, aformosin,

calycosin, pseudobaptigen and fujikinetin, four flavanones, naringenin, eridictyol, hesperetin and hesperidin, three coumestans, medicagol, flemichapparin and sophoracoumestan B, two xanthones, mangiferin and isomangiferin and a single flavone, luteolin were identified. Reliable quantification of the constituents was not possible due to substantial losses as a consequence of extensive enrichment and fractionation procedures required, due to the complexity of the mixture. Relative large quantities of the cyclitol, (+) pinitol, was also present (Ferreira *et al*., 1998).

Subsequent investigations of methanol extracts of fermented leaves and stems of *C. intermedia* (Kamara *et al*., 2003) revealed the presence of additional flavonoids. These compounds included tyrosol and a methoxy analogue, 2-{4-[*O*-α-apiofuranosyl-(1"→6')-β-D-glucopyranosyloxy]phenyl}ethanol,4-[*O*-α–apiofuranosyl–(1"→2')-β-D-glucopyra-nosyloxy] enzaldehyde, five glycosylated flavonols, four flavanones, two isoflavones, wistin and a new diglycosylated isoflavone and two flavones.

Analysis of acetone and methanol extracts prepared from unfermented *C. subternata* uncovered the presense of compounds also found in *C. intermedia* namely (+)-pinitol, hesperidin, luteolin and mangiferin (Kamara *et al*., 2004). Additional compounds present included shikimic acid, *p*-coumaric acid, 4-glucosyltyrosol, epigallocatechin gallate, the isoflavone orobol, the flavanones narirutin and eriocitrin, the flavones 5-deoxyluteolin and scolymoside and the flavonol *C*-6-glucosylkampferol.

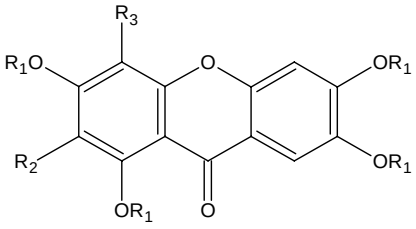
Aqueous extracts of fermented and unfermented *C. sessiliflora* contain higher levels of total polyphenols and flavonoids than fermented and unfermented *C. genistoides, C. subternata, C. maculata* and *C. intermedia,* as determined by Folin-Ciocalteu reactivity (Hubbe & Joubert, 2000a). Richards (2002) confirmed that *C. sessiliflora* contains the highest levels of total polyphenols. The fermentation step during processing has been shown to reduce the total polyphenol content of aqueous extracts of various *Cyclopia spp.* (Hubbe & Joubert, 2000b; Richards, 2002).

Demonstration of the antioxidant activity of aqueous extracts prepared from several *Cyclopia spp.* (Hubbe & Joubert, 2000a; Hubbe & Joubert, 2000b) led to investigation of its use in preparations of antioxidant–rich extracts (Joubert *et al*., 2003). Commercial preparation of these extracts requires high levels of the compounds of interest. This led to quantification of three major polyphenols, mangiferin, isomangiferin and hesperidin in methanol extracts of species currently under investigation for cultivation i.e. *C. intermedia, C. genistoides, C. sessiliflora* including *C. maculata* (Joubert *et al*., 2003), although availability and cultivation of the latter is limited. *C. intermedia* is currently mainly harvested from wild populations, while *C. subternata*

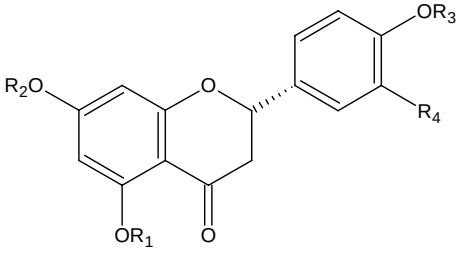
(limited harvesting from wild populations) and *C. genistoides* are the main species cultivated (Dr. E Joubert, ARC Infruitec-Neitvoorbij, Stellenbosch, personal communication, 2004). Reversed-phase HPLC analysis showed that unfermented *C. genistoides* contained the highest levels of the xanthones, mangiferin (3.61 g 100 g^{-1}) and isomangiferin (0.54 g 100 g^{-1}), while unfermented *C. intermedia* contained the highest levels of the flavanone, hesperidin (1.74 g 100 g^{-1}). The lowest levels of mangiferin (1.04 g 100 g^{-1}) and hesperidin (0.29 g 100 g^{-1}) were found in unfermented *C. sessiliflora.* Richards (2002) confirmed that mangiferin and isomangiferin (quantified together) levels were much higher in fermented and unfermented aqueous extracts of *C. genistoides* than in *C. intermedia, C. subternata* and *C. sessiliflora.* Unfermented *C. intermedia* contained the highest levels of hesperidin, while hesperidin levels were similar in fermented *C. intermedia* and *C. subternata* (Richards, 2002).

Variations found in phenolic compostion within the same *Cyclopia* species is attributed to differences in solubility of phenolic compounds, due to the solvents used for analysis, but it has also been postulated that growth and the leaf to stem ratio of the plant material used in production influences the phenolic composition of the final product (Joubert *et al*., 2003). Variations in the polyphenolic content of two types of *C. genistoides* due to date of harvesting have been demonstrated (Joubert *et al*., 2003). Although it did not influence the levels of isomangiferin and hesperidin in unfermented *C. genistoides* significantly, a significant decrease ($P < 0.001$) in the mangiferin content from the end of March to mid-July (period that plant material was harvested) was evident.

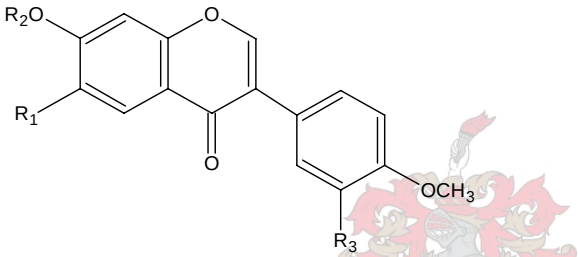
The mangiferin and hesperidin contents of the two types of *C. genistoides* also differed, with the Overberg type containing higher levels of mangiferin (6.37 g 100 g^{-1}) and lower levels of hesperidin (2.23 g 100 g^{-1}) than the West Coast type (4.93 g 100 g^{-1} mangiferin and 5.21 g 100 g^{-1} hesperidin) (Joubert *et al*., 2003). The phenolic compositions of *Cyclopia spp*. is influenced by wide-ranging factors, which afford inclusion of different samples (selected from different plantations and different areas) from the same species essential in investigation of biological properties of honeybush tea.



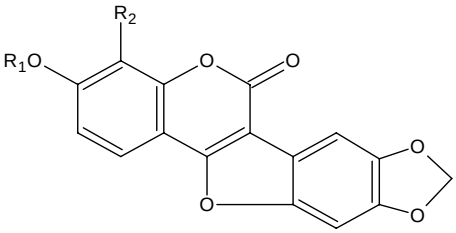
Xanthone
mangiferin R_1=R_3=H,
R_2=2-β-*D*-glucopyranosyl
isomangiferin R_1=R_2=H,
R_3= 2-β-*D*-glucopyranosyl



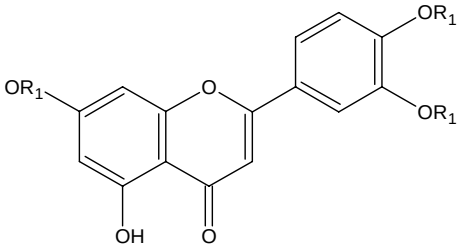
Flavanones
naringenin R_1=R_2=R_3=R_4=H
eriodictyol R_1=R_2=R_3=H, R_4=OH
hesperetin R_1=R_2=H, R3=Me, R_4=OH
hesperidin R_1=H, R_2 = rutinosyl, R_3=Me, R_4=OH
narirutin R_1= R_3= R_4, R_2 = rutinosyl
eriocitrin R_1= R_3= H, R_2 = rutinosyl, R_4=OH



Isoflavones
formononetin R_1=R_2=R_3=H
afrormosin R_1=OMe
calcycosin R_1=R_2=H, R_3=OH



Coumestans
medicagol R_1=R_2=H
flemichapparin R_1=Me, R_2=H
sophoracoumestan B R_1=H, R_2=OMe



Flavone
luteolin R_1=OH

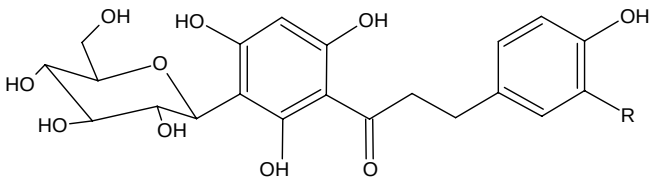
Figure 11 Structures of the major compounds in *C. intermedia, C. subternata, C. genistoides* and *C. sessiliflora*, mangiferin and hesperidin (Joubert *et al*., 2003) and other phenolic compounds that have been isolated from fermented *C. intermedia* (Ferreira *et al*., 1998) and unfermented *C. subternata* (Kamara *et al*., 2004).

Rooibos tea (Aspalathus linearis)

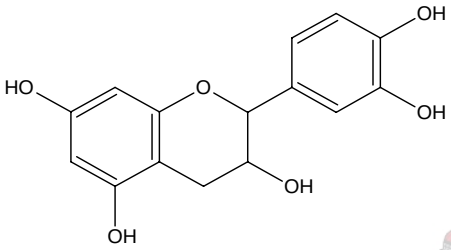
Rooibos tea has an unique flavonoid composition, since it contains aspalathin (2',3,4,4',6'-pentahydroxy-3-*C*-*β*-D-glucopyranosyldihydrochalcone) (Fig. 12), the principle monomeric flavonoid in unprocessed rooibos tea (Koepen & Roux, 1966). Nothofagin (2',3,4,4',6'-tetrahydroxy-3-*C*-*β*-D-glucopyranosyldihydrochalcone) is structurally similar to aspalathin except for the hydroxylation pattern of the B-ring (Fig. 12) and is present in unfermented rooibos tea. Other phenolic compounds present in rooibos tea include the flavones iso-orientin, orientin (Koeppen *et al*., 1962; Koeppen & Roux, 1965), vitexin, iso-vitexin, chrysoeriol (Rabe *et al*., 1994) and luteolin (Snyckers & Salemi, 1974) and the flavonols rutin, isoquercitrin (Koeppen *et al*., 1962) and quercetin (Snyckers & Salemi, 1974) (Fig. 14). Phenolic acids that have been isolated from rooibos tea include *p*-hydroxybenzoic, protocatehuic, vanillic, caffeic, *p*-coumaric and feurulic acids (Rabe *et al*., 1994). The tannin content of the beverage is low in comparison to that of black tea (Blommaert & Steenkamp, 1978).

The dihydrochalcones, aspalathin and nothofagin have been identified as the major polyphenols in unfermented rooibos tea (Joubert, 1996). Quantification of flavonoids from aqueous and methanolic extracts prepared from commercial fermented rooibos tea led to the main compounds being identified as aspalathin, rutin and orientin, followed by iso-orientin and isoquercitin (Bramati *et al*., 2002). Bramati *et al*. (2003) quantified additional flavonoids in unfermented rooibos tea as summarised in Table 4.

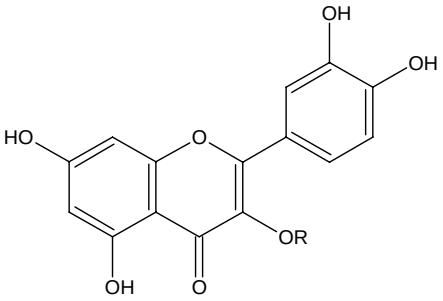
The composition and polyphenolic content of unfermented rooibos differs from that of fermented rooibos tea (Joubert, 1996). Fermentation during processing results in a reduction of the aspalathin and nothofagin content of rooibos tea, with fermented tea containing only about 7% of the dihydrochalcones originally present in the unfermented tea (Joubert, 1996). The aspalathin content of unfermented rooibos samples has been shown to be much higher, between 35 and 68% of the total polyphenol content, than that in fermented samples (Schulz *et al*., 2003). Koeppen & Roux (1966) postulated that aspalathin is converted to 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin (Koeppen & Roux, 1966) and it has been indicated that these conversions possibly occur during fermentation of rooibos tea (Joubert, 1996). The presense of 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin was confirmed by subjecting aspalathin to biocatalytic oxidative cyclisation (Marais *et al*., 1998).



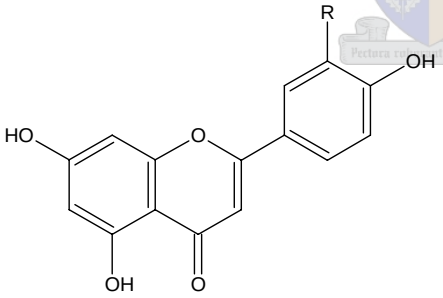
Dihydrochalcones
aspalathin R=OH
nothofagin R=H



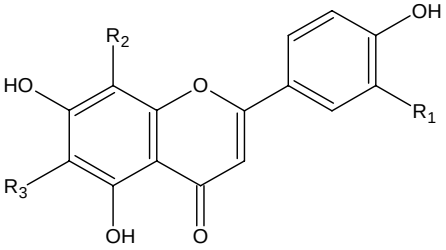
Flavanol
(+) catechin



Flavonol
quercetin R=H
isoquercitrin R= β-D-glucopyranosyl
rutin R= rutinosyl



Flavone
luteolin R=OH
chrysoeriol R=OCH_3



C-C linked flavone glycosides
iso-orientin R_1=OH, R_2= glycopyranosyl
isovitexin R_1=H, R_2= glycopyranosyl
orientin R_1= OH, R_3=glycopyranosyl
vitexin R_1= H, R_3=glycopyranosyl

Figure 12 Structures of aspalathin and nothofagin, the dihydrochalcones present in rooibos tea (Joubert, 1996) and the flavanol (+) catechin, the flavonols quercetin, isoquercitrin and rutin, the flavones chrysoeriol and luteolin, and four C-C linked flavone glycosides, iso-orientin, isovitexin, orientin and vitexin (Rabe *et al*., 1994).

Table 4 Flavonoids detected in aqueous extracts from fermented and unfermented rooibos tea (g 100 g^{-1}) as determined with HPLC analysis using a reversed phase C_{18} column.

Compound	Fermented[a]	Unfermented[b]
Aspalathin	0.123	4.992
Orientin	0.1003	0.234
Isoorientin	0.083	0.357
Vitexin	0.033	0.050
Rutin	0.127	0.169
Isovitexin	0.027	0.066
Isoquercitrin and hyperoside	0.043	0.033
Luteolin	0.003	0.002
Quercetin	0.011	0.004
Chrysoeriol	0.002	0.0008

[a] Bramati *et al*., 2002.
[b] Bramati *et al*., 2003.

The diastereomeric pair of flavanones, (S)- and (R)-eriodictyol-6-*C*-β-D-glucopyranosid, formed through oxidative cyclisation of aspalathin under conditions mimicking the fermentation process (Marais *et al*., 2000). Reduced dihydrochalcone content in fermented tea is attributed to enzymatic and chemical oxidation of polyphenols (Joubert, 1996). Oxidation is initiated during processing when the tea is crushed, leading to cell damage and consequent exposure to oxygen.

Green and black teas (Camellia sinensis)

Camellia sinensis contains many organic constituents of which some appear to have medicinal and health benefits (Balentine *et al*., 1997). Apart from protein and carbohydrates, these organic constituents also include a considerable amount of polyphenols and green tea is regarded as one of the major sources of natural flavonoids (Chen, 1997).

Polyphenols in green tea (unfermented *C. sinensis*) predominantly belongs to three subclasses: the flavanols, the flavonols and the flavones (Balentine *et al*., 1997). The main classes in *Camellia sinensis* are flavanols and flavonols, which constitute approximately 30% of the dry weight of the fresh leaf. The predominant form is catechins (flavan-3-ols), which are characterised by di- or tri-hydroxyl group substitution of the B ring and the meta-5,7-dihydroxy substitution of the A ring. Four major catechins are found in fresh leaves: (-) epicatechin, (-) epicatechin gallate, (-) epigallocatechin and (-) epigallocatechin gallate (Fig. 13). The quantities of different phenolic compounds in green tea are summarised in Table 5. The major green tea flavonols include quercetin, kaempferol, myricitin, and their glycosides, which are characterised by a 4-oxo 3-hydroxy C ring. Flavonol glycosides make up 2 to 3% of the water-soluble extract solids of green tea. Their poor solubility causes the flavonol aglycones not to be found in significant quantities in the green tea beverage. Other constituents of green tea comprise caffeine, theobromine, theophylline and phenolic acids (Gupta *et al*., 2002).

Black tea manufacture involves oxidation of flavanols, by polyphenol oxidase to form the reddish-brown theaflavins and thearubigins (Bu-Abbas *et al*., 1997). The conversion of colourless catechins to a range of products of orange-yellow to red-brown colour occur through a series of oxidative condensation reactions, and numerous volatile flavour constituents are formed (Balentine *et al*., 1997). The levels of different phenolic compounds present in black tea are summarised in Table 6. Despite the fact that the thearubigins are the most abundant polyphenolic oxidation products in black tea, very little is known about the chemistry of these compounds (Haslam, 2003).

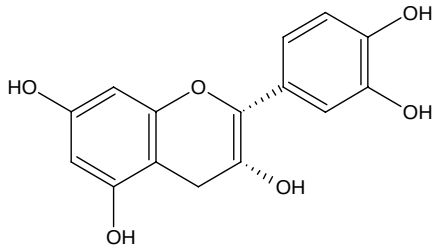
Table 5 Phenolic content of green tea (*Camellia sinensis*) as determined by different researchers and methods.

Compound	Level (% w/w)
(-) Epigallocatechin gallate (EGCG)	3.15–7.48[a], 10.55[b], 2.86–3.38[c], 2.19–3.95[d]
(-) Epigallocatechin (EGC)	3.10– 6.10[a], 2.35[b], 2.91– 3.79[c], 2.75–5.02
(-) Epicatechin gallate (ECG)	0.76–1.88[a], 2.75[b], 0.43–0.58[c], 0.50– 0.91[d]
(-) Epicatechin (EC)	0.76–1.30[a], 0.63[b], 0.8–1.01[c], 0.96–2.00[d]
(-) Catechin (C)	0.43–0.93 [a], 0.35[b]
(-) Catechin gallate (CG)	0.10–0.29[a]
Flavanols (Catechins)	30–42[a], 17–30 [b]
Flavonols + flavonol glycosides	3–4[b]

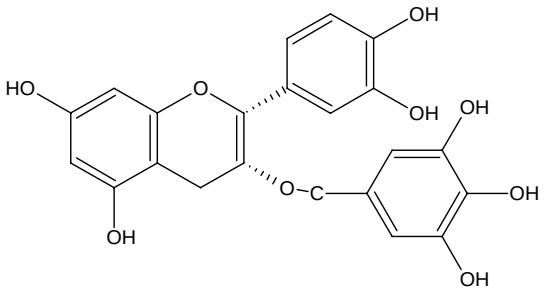
[a] Seasonal variation in the polyphenol content of unprocessed tea shoots. Polyphenols were separated by two-way chromatography with estimations based on the measurements of their extinction coefficients at 275 nm (Bhatia & Ullah, 1968).
[b] Polyphenol content of green tea leaves quantified by HPLC analysis, as resported by Lunder (1992).
[c] Polyphenol content quantified by HPLC analysis, in three different kinds of tea, Ureshino-cha, Yame-cha and Tiran-cha (Kumamoto *et al*., 2000).
[d] Variation in individual flavanol concentration in nine commercial green teas extracted using water (80 ˚C) as determined by HPLC analysis (Price & Spitzer, 1993).

Table 6 Phenolic composition of black tea (fermented *Camellia sinensis*) (Lunder, 1992).

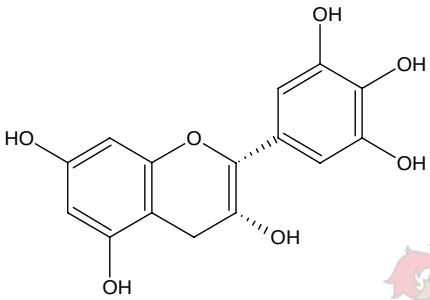
Compound	Level (% w/w)
Thearubigins	15-20
Theaflavins	1-2
Bisflavonols	2-4
Phenolic acids	4
Unchanged flavanols	1-3
Flavonols and flavonol glycosides	2-3



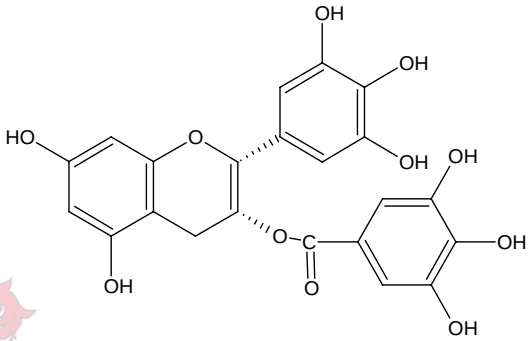
(-) Epicatechin



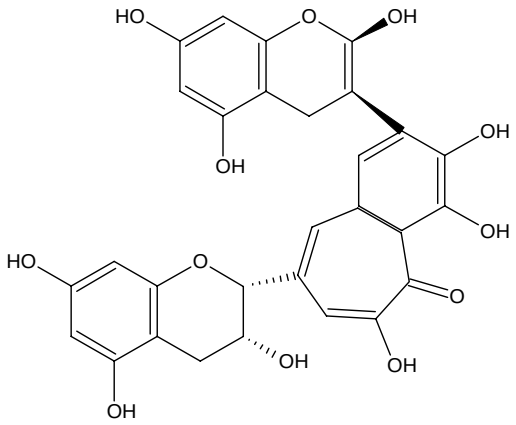
(-) Epicatechin gallate



(-) Epigallocatechin



(-) Epigallocatechin gallate



Theaflavin

Figure 13 The structures of the major catechins in green tea and theaflavin in black tea (Yang *et al*., 2000).

It is estimated that up to 75% of the phenolic flavan-3-ol substrates may be converted to thearubigins during the manufacture of black tea. Four main theaflavins (theaflavin, theaflavin 3-gallate, theaflavin 3'gallate and theaflavin di-gallate) and two groups of minor theaflavins, isotheaflavins and neotheaflavins, are found in black tea (Fig.13) (Balentine, 1996). The amount of catechins in various tea extracts differ and are as follows: green (26.7%) > oolong (23.2%) > pouchong (15.8%) and black tea (4.3%) (Yen & Chen, 1996). The caffeine and phenolic content of oolong tea, 8.3% and 32.4% respectively, has however been found to be higher than green and black tea (Yen & Chen, 1996).

Variation in the chemical composition of tea leaves is attributed to environmental effects, age of the leaves, rate of growth, varietal differences, methods of processing, harvesting season and modes of propagation (Robertson, 1992; Yen & Chen, 1996; Gupta *et al*., 2002). The flavanol content of var. *sinensis* is approximately half of that in var. *asamica* (Takeo, 1992). The much greater flavanol content of var. *asamica* renders it unfit for use in green tea production, since it results in excessive bitterness. The flavanol content of var. *sinensis* is just sufficient to result in acceptable astringency for production of the green tea beverage (Takeo, 1992).

Antimutagenic acitivity of tea

Estimation of antimutagenic activity

The *Salmonella* mutagencity assay, designed specifically for detection of chemically induced mutagenesis (Ames *et al*., 1975), has been recognised by government agencies and corporations, which led to worldwide use as an initial screening method to determine the mutagenic potential of new chemicals and drugs (Mortelmans & Zeiger, 2000). The test is based on the use of several histidine dependent *Salmonella* strains with different mutations in various genes to detect mutagens, since only the bacteria that revert to histidine independence (his^+) are able to grow on minimal agar containing a trace of histidine. International guidelines have been developed by institutions such as the Organisation for Economic Co-operation and Development (OECD) and International Commission on Harmonisation (ICH) to ensure uniformity of testing procedures of chemicals, like drugs and biocides, before submission of data to regulatory agencies for registration or acceptance (Mortelmans & Zeiger, 2000). Bacteria lack the ability to metabolise chemicals via cytochrome P450, rendering inclusion of an exogenous mammalian metabolic activation system a key component in the development of the assay (Ames *et al*.,1973). Although other tissues and species can be used, rat liver has been found to be the most

convenient source of activating enzymes for use in the *Salmonella* mutagenicity assay (Maron & Ames, 1983). The metabolic activation system, referred to as the S9 mix, include a 9 000 *x g* supernatant fraction of a rat liver homogenate (S9 microsomal fraction), NADPH and co-factors for NADPH-supported oxidation (Maron & Ames, 1983). Pretreatment of animals with mixed function oxidase inducers such as Aroclor 1254, phenobarbital and β-naphthoflavone is used to increase the level of metabolising enzymes (Mortelmans & Zeiger, 2000).

Other developments that contributed to reduced cost and easier performance included the plate incorporation assay protocol developed to replace spot test or liquid suspension procedures (Mortelmans & Zeiger, 2000).

The *Salmonella* strains used for mutagenicity testing are all histidine dependent due to a mutation in the histidine operon (Mortelmans & Zeiger, 2000). The tester strains recommended for general mutagenicity testing are TA97, TA98, TA100, TA102, TA1535 and TA1538 (Maron & Ames, 1983).

Tester strains TA100 and TA1535 contain the *hisG46* mutation in the *hisG* gene, which codes for the first gene of histidine biosynthesis (Ames, 1971). This mutation causes substitution of $^{-GGG}_{CCC-}$ (proline) for $^{-GAG}_{GTC-}$ (leucine) in the wild type organism. TA100 and TA1535 are mainly used to detect mutagens that cause base-pair substitutions, principally at one of these G-C pairs (Mortelmans & Zeiger, 2000). Tester strains TA1538 and its R-factor derivative TA98 contain the *hisD3052* mutation in the *hisD* gene, which is a –1 frameshift mutation (Maron & Ames, 1983) and affect the reading frame of the repetitive –C-G-C-G-C-G-C-G- sequence (Isono & Yourno, 1974). Various frameshift mutagens, such as 2-acetylaminofluorene (2-AAF) and several aromatic nitroso derivatives of amine carcinogens contain the ability to induce the reversion of the *hisD3052* mutation (Mortelmans & Zeiger, 2000). The number of spontaneously induced revertant colonies is relatively constant for each strain (Mortelmans & Zeiger, 2000).

Additional mutations and genetic alterations that are used to make tester strains more sensitive to chemical mutagens include a deletion mutation through the *uvrB-bio* genes, *rfa-* mutation, introduction of plasmid pKM101 (R-factor) and insertion of the mutation *hisG428* on the multi-copy plasmid pAQ1 (Mortelmans & Zeiger, 2000).

Antimutagenic activity of tea extracts and some of their major polyphenols

The antimutagenic activity of tea extracts and some of their major phenolic constituents in the *Salmonella* mutagenicity assay, reviewed herewith, is summarised in Tables 7-10. Antimutagenicity of green tea is the subject of significantly more studies than for oolong and black teas. Water extracts of green tea and green tea polyphenols have been found to significantly inhibit the reverse mutation induced by B[a]P, AFB_1 (Table 7), 2-aminofluorene (2-AF) (Table 7) and methanol extracts of coal tar pitch in the *Salmonella* mutagenicity assay using tester strains TA100 and TA98 in the presence of rat-liver microsomal activation system (Wang *et al*., 1989).

The antimutagenic action of tea (*Camellia sinensis*) may vary with the extent of fermentation during the manufacturing of tea (Yen & Chen, 1996; Chen & Yen, 1997). It has been demonstrated that the antimutagenic activity of semifermented tea (oolong) is greater than that of fermented tea (black) and unfermented tea (green) (Table 7) (Yen & Chen, 1994; Yen & Chen, 1995; Yen & Chen, 1996). The higher antimutagenic activity of oolong tea (semi-fermented) compared to that of black (fermented) and green (unfermented) teas, was attributed to possible formation of antimutagenic substances during manufacturing processes of *Camellia sinensis* (Yen & Chen, 1994).

A good correlation has been reported for the antioxidant and antimutagenic activity of tea extracts from green, pouching, oolong and black tea, but variation with the mutagen and antioxidative activity in different assays was evident (Yen & Chen, 1995). Tea extracts possessed strong antimutagenic activity against five indirect acting mutagens i.e. AFB_1 (Table 7), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1), benzo[a]pyrene (B[a]P) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (Yen & Chen, 1995). Black tea demonstrated the weakest protective effect against AFB_1-induced mutagenesis using both tester strain TA100 and TA98 in the *Salmonella* mutagenicity assay. The inhibitory effect of oolong tea against AFB_1 induced mutagenesis was greater towards tester strain TA98 than TA100 (Yen & Chen, 1995).

In a subsequent study it was demonstrated that the inhibitory effect of tea extracts against IQ and Glu-P-1 mutagenesis using TA100 demonstrated significant correlation to the contents of catechins and ascorbic acid in green, oolong and black teas (Yen & Chen, 1996). Antimutagenic activity of tea extracts to Trp-P-1 in TA98 and TA100 was well correlated to the caffeine contents, while no significant correlation was found between antimutagenicity of tea extracts against B[a]P and AFB_1 in TA100 and the content of major components in tea extracts (Yen &

Chen, 1996). It was postulated that antimutagenic action of tea extracts (*Camellia sinensis*) against IQ and B[a]P are due to a combination of distinctive mechanisms (Chen & Yen, 1997). The results of this study suggested that inhibition of cytochrome P450 mediated metabolism of IQ and B[a]P to ultimate mutagenic metabolites and interaction of tea with promutagens and their metabolites, are responsible for the reduced mutagenic potential (Chen & Yen, 1997).

Yamada & Tomita (1994) found that water extracts of the leaves of black and oolong tea significantly decrease the mutations induced by Trp-P-1, Glu-P-1 and B[a]P (Table 8) in *Salmonella* tester strains TA98 and TA100 in the presense of a rat liver microsomal activation system.

Regardless of the differences in the flavanol content of green and black tea, they possess similar antimutagenic activity against a number of indirect-acting mutagens (Bu-Abbas *et al*., 1996). This indicates that flavanols are unlikely to be the principal component of tea expressing its antimutagenic acitivity. In a succeeding study to evaluate the role of individual flavanols in the antimutagenic potential of green tea through investigation of extracts of green tea and four fractions (fraction A-D, Table 8), no relationship could be established between the flavanol content and antimutagenic potential (Bu-Abbas *et al*., 1997). The ability of each fraction to inhibit the mutagenicity of the four model mutagens N-nitrosopyrrolidine, B[a]P (Table 8), 2-aminoanthracene and Glu-P-1 (2-amino-6-methyldipyrido[1,2-*a*3,2-*d*]imidazole) was investigated in the *Salmonella* mutagenicity assay. Although fractions A and C had the highest flavanol content and fraction B had the lowest flavanol content, but the highest theogallin content, fractions C and D demonstrated the highest antimutagenic acitivity against B[a]P (Table 8). It was concluded that components other than flavanols are the major antimutagenic factors present in green tea (Bu-Abbas *et al*., 1997).

Comparison of the antimutagenic properties of aqueous extracts prepared from fermented and unfermented rooibos tea to that of green and black tea showed that green tea had the strongest antimutagenic activity against 2-AAF (Standley, 1999). Unfermented rooibos tea exhibited higher antimutagenic activity than black and fermented rooibos tea (Table 8). Marnewick *et al.* (2000) reported that aqueous extracts prepared from fermented and unfermented rooibos and honeybush tea (*C. intermedia*) possess antimutagenic activity against 2-AAF and AFB_1 induced mutagenesis in the *Salmonella* mutagenicity assay using tester strains TA98 and TA100 (Table 9). Inhibition of direct acting mutagens, methyl methanesulfonate (MMS, cumolhydroperoxide (CHP), and hydrogen peroxide (H_2O_2) with tester strain TA102 (strain designed to detect oxidative mutagens and carcinogens), was found to be far less.

Antimutagenic and antioxidant activity of rooibos tea was assessed using samples collected from each of its major processing stages (Standley *et al*., 2001). The processing stages included unfermented tea, fermented tea, fermented sun-dried tea, tea before steam pasteurisation and tea after steam pasteurisation. The reduction in the polyphenolic content of the tea as a result of fermentation has been associated with decreased antioxidant and antimutagenic effects 2-AAF (Table 9) (Standley *et al*., 2001). It is, however, not known whether the compounds responsible for the antimutagenic activity are similar to those exhibiting antioxidant activity (Standley *et al*., 2001). Methods used to determine antioxidant activity have also been implicated in differences in the measured antioxidant potency (Standley *et al*., 2001). This has also been demonstrated in *Camellia sinensis* teas. Yen & Chen (1995) reported that antimutagenicity of extracts of *Camellia sinensis* (green, pouching, oolong and black tea) correlated well with antioxidant effects, although it varies with the mutagen used and the antioxidant properties.

Richards (2002) demonstrated the antimutagenic activity of aqueous extracts of four species of fermented and unfermented honeybush i.e. *Cyclopia intermedia, C. sessiliflora, C. genistoides* and *C. subternata* against 2-AAF, with the exception of unfermented *C. genistoides* which enhanced mutagenicity in the *Salmonella* mutagenicity assay (Table 10). Unfermented *C. genistoides* was however not mutagenic in the presence or absence of S9 in the *Salmonella* mutagenicity assay using tester strain TA98 (Richards, 2002).

The phenolic compounds, mangiferin, hesperidin, hesperetin, eridictyol and luteolin present in *Cyclopia spp.*, demonstrated different effects against 2-AAF-induced mutagenesis in the *Salmonella* mutagenicity assay using tester strain TA98 (Table 10) (Richards, 2002). Luteolin exhibited very high antimutagenic activity (87%), while hesperetin (9%) and mangiferin (8%) were not very effective inhibitors of 2-AAF mutagenesis (Table 10). Eriodictyol and hesperedin appeared to enhance the mutagenicity of metabolically activated 2-AAF (Richards, 2002).

Table 7 Antimutagenic activity of tea extracts prepared from black, oolong and green teas, as well as (-) epigallocatechin gallate, against different metabolically activated mutagens in the *Salmonella* mutagenicity assay using tester strain TA98 and TA100.

Extract/compound (concentration per plate)	Mutagen	Concentration per plate	Strain	Activity	Reference
Aqueous green tea extracts (1000 µg/plate)	AFB_1	1 µg/plate	TA100	65% inhibition of mutagenicity	Wang *et al*., 1989
Aqueous green tea extracts (1000 µg/plate)	AFB_1	1 µg/plate	TA98	91% inhibition of mutagenicity	
Green tea polyphenols (extracted with 80% ethanol) (100 µg/plate)	AFB_1	na[a]	TA98	77% inhibition of mutagenicity	
(-) Epigallocatechin gallate (100 µg/plate)	AFB_1	na[a]	TA98	91% inhibition of mutagenicity	
Green tea polyphenols (100 µg/plate)	2-AF[b]	10 µg/plate	TA98	87% inhibition of mutagenicity	
(-) Epigallocatechin gallate (100 µg/plate)	2-AF[b]	10 µg/plate	TA98	96% inhibition of mutagenicity	
Aqueous green tea extract (0.7 mg/plate)	AFB_1	0.5 µg/plate	TA98	96% inhibition of mutagenicity	Yen & Chen, 1995
Aqueous oolong tea extract (0.7 mg/plate)	AFB_1	0.5 µg/plate	TA98	92% inhibition of mutagenicity	
Aqueous black tea extract (0.7 mg/plate)	AFB_1	0.5 µg/plate	TA98	73% inhibition of mutagenicity	
Aqueous green tea extracts (0.7 mg/plate)	AFB_1	0.5 µg/plate	TA100	86% inhibition of mutagenicity	
Aqueous oolong tea extracts (0.7 mg/plate)	AFB_1	0.5 µg/plate	TA100	91% inhibition of mutagenicity	
Aqueous black tea extracts (0.7 mg/plate)	AFB_1	0.5 µg/plate	TA100	87% inhibition of mutagenicity	

[a] not available
[b] 2-aminofluorene

Table 8 Antimutagenic activity of aqueous extracts prepared from black, oolong and green teas as well as fermented and unfermented rooibos tea, against different metabolically activated mutagens in the *Salmonella* mutagenicity assay using tester strain TA98 and TA100.

Extract/compound (concentration per plate)	**Mutagen**	**Concentration per plate**	**Strain**	**Activity**	**Reference**
Black tea 500 μg/plate	Benzo[a] pyrene	10 μg/ plate	TA98	88% inhibition of mutagenicity	Yamada & Tomita, 1994
Black tea 1000 μg/plate	Benzo[a] pyrene	10 μg/ plate	TA98	99% inhibition of mutagenicity	
Oolong tea 500 μg/plate	Benzo[a] pyrene	10 μg/ plate	TA98	75% inhibition of mutagenicity	
Oolong tea 1000 μg/plate	Benzo[a] pyrene	10 μg/ plate	TA98	95% inhibition of mutagenicity	
Green tea (7.43 mg mL^{-1} total solids)	Benzo[a] pyrene	25 μg/ plate	TA 100	67% inhibition of mutagenicity	Bu-Abbas *et al*., 1997
Fraction A (2.96 mg mL^{-1} total solids)	Benzo[a] pyrene	25 μg/ plate	TA 100	69% inhibition of mutagenicity	
Fraction B (1.09 mg mL^{-1} total solids)	Benzo[a] pyrene	25 μg/ plate	TA 100	61% inhibition of mutagenicity	
Fraction C (1.56 mg mL^{-1} total solids)	Benzo[a] pyrene	25 μg/ plate	TA 100	81% inhibition of mutagenicity	
Fraction D (1.26 mg mL^{-1} total solids)	Benzo[a] pyrene	25 μg/ plate	TA 100	80% inhibition of mutagenicity	
Green tea (1.25 mg/plate)	2-AAF	2.5 μg/ plate	TA98	67% inhibition of mutagenicity	Standley, 1999
Black tea (1.25 mg/plate)	2-AAF	2.5 μg/ plate	TA98	46% inhibition of mutagenicity	
Fermented rooibos tea (1.25 mg/plate)	2-AAF	2.5 μg/ plate	TA98	47% inhibition of mutagenicity	
Unfermented rooibos tea (1.25 mg/plate)	2-AAF	2.5 μg/ plate	TA98	59% inhibition of mutagenicity	

Table 9 Antimutagenic activities of aqueous extracts prepared from fermented and unfermented rooibos tea and *Cyclopia spp.* against different metabolically activated mutagens in the *Salmonella* mutagenicity assay using tester strain TA98 and TA100.

Extract/compound (concentration per plate)	Mutagen	Concentration per plate	Strain	Activity	Reference
Fermented rooibos tea (1.49 mg total polyphenols/plate)	2-AAF	5 µg/plate	TA98	75% inhibition of mutagenicity	Marnewick *et al*., 2000
Fermented rooibos tea (2.97 mg total polyphenols/plate)	2-AAF	5 µg/plate	TA98	88% inhibition of mutagenicity	
Unfermented rooibos tea (2.06 mg total polyphenols/plate)	2-AAF	5 µg/plate	TA98	50% inhibition of mutagenicity	
Unfermented rooibos tea (4.12 mg total polyphenols/plate)	2-AAF	5 µg/plate	TA98	97% inhibition of mutagenicity	
Fermented honeybush tea (*C. intermedia*) (0.99 mg total polyphenols/plate)	AFB_1	10 ng/plate	TA100	41% inhibition of mutagenicity	
Fermented honeybush tea (*C. intermedia*) (1.98 mg of total polyphenols/plate)	AFB_1	10 ng/plate	TA100	68% inhibition of mutagenicity	
Unfermented honeybush tea (*C. intermedia*) (1.78 mg total polyphenols/plate)	AFB_1	10 ng/plate	TA100	71% inhibition of mutagenicity	
Unfermented honeybush tea (3.55 mg total polyphenols/plate)	AFB_1	10 ng/plate	TA100	93% inhibition of mutagenicity	
Unfermented rooibos tea (2.5 mg mL^{-1} soluble solids)	2-AAF	2.5 µg/ plate	TA98	88% inhibition of mutagenicity	Standley *et al*., 2001
Fermented rooibos tea (2.5 mg mL^{-1} soluble solids)	2-AAF	2.5 µg/ plate	TA98	60% inhibition of mutagenicity	
Sundried rooibos tea (2.5 mg mL^{-1} soluble solids)	2-AAF	2.5 µg/ plate	TA98	53% inhibition of mutagenicity	
Sieved rooibos tea (2.5 mg mL^{-1} soluble solids)	2-AAF	2.5 µg/ plate	TA98	48% inhibition of mutagenicity	
Steam pasteurised rooibos (2.5 mg mL^{-1} soluble solids)	2-AAF	2.5 µg/ plate	TA98	45% inhibition of mutagenicity	

Table 10 Antimutagenic activity of aqueous extracts prepared from fermented and unfermented *Cyclopia spp.* and phenolic compounds present in these species, against 2-AAF in the *Salmonella* mutagenicity assay using tester strain TA98.

Extract/compound	Mutagen	Concentration per plate	Strain	Activity	Reference
Fermented *C. intermedia* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	51% inhibition of mutagenicity	Richards, 2002
Unfermented *C. intermedia* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	64% inhibition of mutagenicity	
Fermented *C. subternata* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	69% inhibition of mutagenicity	
Unfermented *C. subternata* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	48% inhibition of mutagenicity	
Fermented *C. sessiliflora* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	75% inhibition of mutagenicity	
Unfermented *C. sessiliflora* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	61% inhibition of mutagenicity	
Fermented *C. genistoides* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	47% inhibition of mutagenicity	
Unfermented *C. genistoides* (2 mg/plate)	2-AAF	5 µg/ plate	TA98	**-16**% inhibition of mutagenicity	
Mangiferin (0.3 mM/plate)	2-AAF	5 µg/ plate	TA98	8% inhibition of mutagenicity	
Luteolin (0.3 mM/plate)	2-AAF	5 µg/ plate	TA98	87% inhibition of mutagenicity	
Hesperidin (0.3 mM/plate)	2-AAF	5 µg/ plate	TA98	**-9**% inhibition of mutagenicity	
Hesperetin (0.3 mM/plate)	2-AAF	5 µg/ plate	TA98	9% inhibition of mutagenicity	
Eridictyol (0.3 mM/plate)	2-AAF	5 µg/ plate	TA98	**-60**% inhibition of mutagenicity	

Mechanisms of antimutagenicity

Structure criteria for effective antimutagenic activity of flavonoids

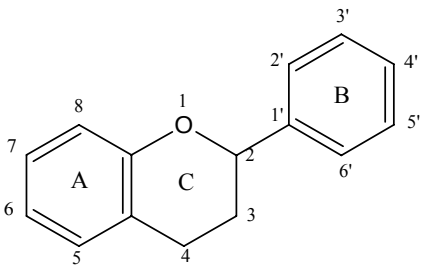
Distinct structure-antimutagenicity relationships against several mutagens have been demonstrated for several flavonoids (Francis *et al*., 1989; Edenharder *et al*., 1993; Das *et al*., 1994; Edenharder & Tang, 1997; Edenharder *et al*., 1997). The physiological activities of flavonoids are strongly affected by their glycosylation and hydroxylation pattern as well as the type of C2-C3 bond (Das *et al*., 1994). A slight change in the chemical structure may result in varying degrees of reactivity and a change in the pattern of glycosylation, hydroxylation and methylation or the presence or absence of the C2-C3 double bond leads to variations in solubility, absorptivity, resonance contributions, ionisation constants, metal chelation and thus biological activity (Das *et al*., 1994).

In vitro antimutagenic activity of flavonoids may vary when determined against different mutagens and different *Salmonella* strains. Although no fundamental differences are detected between TA100 (base pair substitution) and TA98 (frameshift mutations) in inhibition of mutagenicity of cooked food mutagens by flavonoids (Edenharder *et al*., 1993), antimutagenic activity of flavanones against AFB_1 shows a strain specific response (Francis *et al*., 1989).

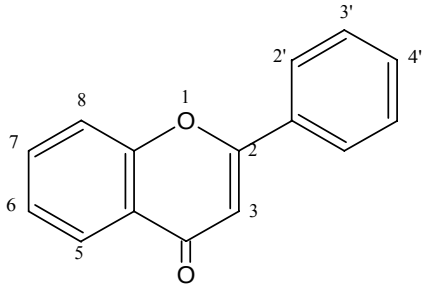
(i) Carbonyl function at C4 of the flavane nucleus

Flavonoids without a carbonyl function at C4 do not have antimutagenic activity against heterocylic amines (Edenharder *et al*., 1993) or nitroarenes (Edenharder & Tang, 1997) in the *Salmonella* mutagenicity assay with tester strain TA98. Flavones, flavonols and flavonones containing the 4-ketogroup have antimutagenic activity against cooked food mutagens, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline (MeIQx), 2-amino-6-methyl-dipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) in *Salmonella typhimurium* TA98 (Edenharder *et al*., 1993). Flavonols and anthocyanidins lacking the keto-group at C4 are inactive against the above mentioned cooked food mutagens (Edenharder *et al*., 1993).

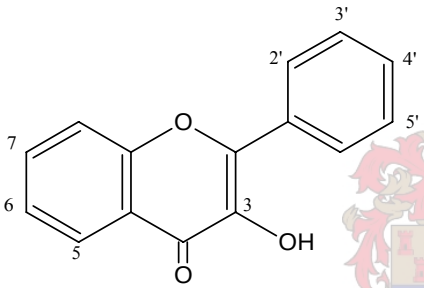
The keto function at C4 is also essential for antimutagenic activity against nitroarenes, 2-nitrofluorene (2-NF), 3-nitrofluoranthene (3-NFA) and 1-nitropyrene (1-NP) with *Salmonella typhimurium* TA98 (Edenharder & Tang, 1997).



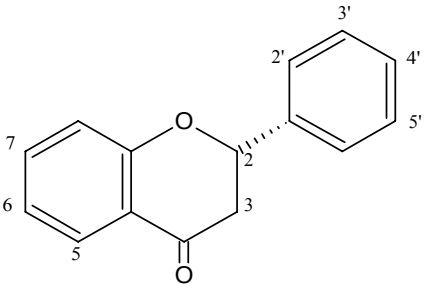
Flavonoid



Flavone



Flavonol



Flavanone

Figure 14 The nuclear structure of flavonoids and the chemical structure of flavone, flavonol and flavanone.

This was demonstrated by absence of antimutagenic activity in flavanols (catechins) and anthocyanidins where this keto group is not present and potent antimutagenic activity was demonstrated by flavones, flavanones, flavonols, isofavonoids and chalcones containing a carbonyl function at C4 (Edenharder & Tang, 1997).

(ii) *Glycosylation*

Investigating antimutagenic activity of flavonoids against heterocyclic amines (Edenharder *et al*., 1993) and nitroarenes (Edenharder & Tang, 1997), demonstrated that most glycosides are not active antimutagens against both types of mutagens. Apart from two moderately effective 7-glycosides, eleven flavonoid glycosides have no or weak antimutagenic activity against IQ in *Salmonella typhimurium* TA98 (Edenharder *et al*., 1993). The reduced or abolished antimutagenic acitivity can possibly be explained by attachment of large polar substituents (glycosides) to flavonoids (Edenharder *et al*., 1993).

This is also true for antimutagenic acitivity against nitroarenes (2-NF, 1-NP and 3-NFA), since seven of the eleven flavonoid glycosides investigated for animutagenic activity were completely inactive (Edenharder & Tang, 1997). Antimutagenic activity of the glycosides against nitroarenes was found to be less than that of the corresponding aglycones (Edenharder & Tang, 1997). Interestingly the flavonols morin and fisetin and the glycoside rutin exhibit similar inhibition of AFB_1 induced mutagenesis in *Salmonella typhimurium* TA98 and TA100 (Francis *et al*., 1989).

(iii) Double bond between carbon 2 and 3

The C2-C3 double bond influences antimutagenic activity of flavonoids against IQ in *Salmonella typhimurium* TA98 (Edenharder *et al*., 1993). This is indicated by comparison of antimutagenic potency of corresponding flavones (double bond present) and flavanones (double bond absent). Flavanones have higher inhibitory dose for 50% reduction of mutagenic activity (ID_{50}) values and are thus less antimutagenic than flavones, demonstrating that the C2-C3 double bond results in increased antimutagenic potency against IQ (Edenharder *et al*., 1993).

The C2-C3 double bond also influences the antimutagenic activity of flavones and flavanones against nitroarene induced mutagenesis (Edenharder & Tang, 1997). Naringenin is the only flavanone showing antimutagenic activity against 2-NF, while almost all the tested flavones have relatively good antimutagenic activity against this mutagen.

(iv) The C ring of the flavonoid nucleus

The C ring of the flavonoid nucleus is not a prerequisite for antimutagenic activity against IQ, but results in increased antimutagenic potency (Edenharder *et al*., 1993). This is demonstrated by the fact that chalcone has antimutagenic activity against IQ in *Salmonella typhimurium* TA98, but is less potent than flavones and flavonols. It has also been found that the C ring is not essential for antimutagenic activity against nitroarenes, since chalcone also possesses antimutagenic activity against these mutagens (Edenharder & Tang, 1997).

(v) Polarity, hydroxyl and methoxyl goups

Hydrophobicity of a flavonoid molecule is an important factor in antimutagenic activity against IQ in *Salmonella typhimurium* TA98 (Edenharder *et al*., 1993). Interference of hydrophobicity with antimutagenic potency is demonstrated by introduction of hydroxyl groups leading to reduced antimutagenic activity of the parent compounds flavone, flavonol and flavanone as well as chalcone, against IQ (Edenharder *et al*., 1993). The position of hydroxylation influences antimutagenic acitivity, since hydroxyl substitution at C6 and C2' results in considerably reduced antimutagenic activity of flavones, flavanones and flavonols (quercetagetin and morin) against IQ (Edenharder *et al*., 1993). An increased number of hydroxyl functions resulted in higher ID_{50} values for antimutagenicity of flavonols and flavanones against IQ, but reducing the polarity of hydroxyflavonoids by methylation of the hydroxyl functions always resulted in lower ID_{50} values (Edenharder *et al*., 1993).

Antimutagenic activity of flavones, flavanones and flavonols against nitroarenes in *Salmonella typhimurium* TA98 correlates with the presence of polar hydroxyl groups (Edenharder & Tang, 1997). The parent compound of the flavones is inactive, while a single hydroxyl function at C5, C6 and C7 generates an active compound against 2-NF, 1-NP and 3-NFA induced mutagenesis, with the position of the hydroxyl group having a distinct influence (Edenharder & Tang, 1997). The influence of the number of hydroxyl groups on antimutagenic activity of flavones against nitroarenes, is demonstrated by antimutagenic activity of luteolin (4 OH groups) being higher than apigenin (three OH groups), which has higher antimutagenic activity than chrysin (two OH groups) (Edenharder & Tang, 1997). Inhibition of 3-NFA induced mutagenesis is an exception, since apigenin is slightly more antimutagenic than luteolin.

The parent compound of the flavonols (3-hydroxyflavone) is a very potent antimutagen against nitroarenes (Edenharder & Tang, 1997). Increasing the number of hydroxyl functions of flavonols results in inactive compounds such as morin (5 OH-groups), myricetin (6 OH groups)

and quercetagin (6 OH groups), with the exception of kaempferol (four OH groups), which possesses antimutagenic activity aginst nitroarenes (Edenharder & Tang, 1997). Conversely an increased number of hydroxyl functions in flavanones results in increased antimutagenic potency against nitroarenes (Edenharder & Tang, 1997). This is evident from the absence of antimutagenic activity of flavanone and monohydroxyflavanones and potent antmutagenic activity of naringenin (3 OH groups) and hesperetin (3 OH groups) against nitroarenes (Edenharder & Tang, 1997). Chalcone was a potent antimutagen against nitroarenes, despite the absence of polar functions. The dihydrochalcone, phloretin (4 OH groups) also has moderate antimutagenic activity against nitroarenes (Edenharder & Tang, 1997).

Reduced polarity of hydroxyflavonoids by methylation of the hydroxyl functions results in reduced ID_{50} values, indicating increased antimutagenic potency against IQ (Edenharder *et al*., 1993). On the contrary, reduction of the polarity of hydroxyflavonoids by methylation of hydroxyl functions results in decreased antimutagenic potency against nitroarenes (Edenharder & Tang, 1997).

Specific structure-activity relationships exist for different mutagens with partial overlapping of patterns, which in part certainly originate from substrate specificities of the various mono-oxygenase enzymes, such as cytochrome P450 (Edenharder & Tang, 1997).

Interaction of flavonoids with cytochrome P450

Antimutagenic and anticarcinogenic effects of naturally occurring plant phenols are assumed to be the result of (i) scavenging of the reactive molecular species of carcinogenic metabolites to prevent their reaching the critical target sites (De Flora , 1998) or (ii) interaction with the cytochrome P450 activation system, leading to reduced formation of the ultimate carcinogenic metabolite (Das *et al*., 1985; Das *et al*., 1987; De Flora, 1998)

Kada *et al.* (1985), assumed that the antimutagenicity of EGCG is due to a specific interaction of EGCG with DNA-polymerase III, leading to possible improvement of the fidelity of DNA replication. Wang *et al*. (1987), however, found that tea polyphenols may inhibit the activation of mutagens and carcinogens by interacting with microsomal cytochrome P450 enzyme protein and impairing electron transfer.

Wang *et al*. (1989) reported that there appears to be a relationship between the chemical structure of (-) epicatechin derivatives and interaction with cytochrome P450 and the effect of these compounds on monooxygenase activites (Wang *et al*., 1989). The tendency in both cases appear to be EGCG > ECG > EGC > EC > (+) C with a possible explanation for EGCG and

ECG being more active, being that it contains a pyrogallic moiety and have two active sites: a galloyl and a di- or tri-hydroxyphenyl group (Wang *et al*., 1989).

Interaction of rat liver microsomal cytochrome P450 with tea polyphenols was investigated by observing the change in difference spectra upon addition of (-) epicatechin derivatives and compared to Type II difference spectra (induced by aniline). Addition of the phenolic compounds to microsomal rat liver (prepared from phenobarbital-treated rats) resulted in a spectral change typical of modified Type II (reverse Type I) binding difference spectra, with maximum absorbance at 420 nm and minimum at 380 nm. EGCG and ECG produced greater spectral changes than EGC and EC.

The competition between carbon monoxide and (-) epicatechin derivatives was also examined (Wang *et al*., 1987). Examination of the structure activity relationship of (-) epicatechin derivatives indicated that potency of inhibition was dependent on substitutions at the 2,3-position of 5,7-dihydroxy-benzoflavan and on the number of hydroxyl groups present in these substituents. It was postulated that the observed inhibition of enzyme activities was in part due to binding of the phenolic groups to the catalytic sites of cytochrome P450 (Wang *et al*., 1987). Any alteration in the structure and function of cytochrome P450 may result in altered rates and differential pathways of metabolism of mutagen and carcinogens. Antimutagenic and anticarcinogenic activity of the phenolic flavonoids quercitin and myricetin through inhibition of the activation of carcinogens by interaction with microsomal enzyme systems, is an example *(*Das *et al.,* 1987*).*

Flavonoids have been shown to inhibit activation of IQ in rat liver microsomes (Edenharder *et al*., 1997). The activity of 7-methoxyresorufin-*O*-dealkylase (MROD) and 7-ethoxyresorufin-*O*-dealkylase (EROD) linked to cytochrome P450-dependent 1A1 and 1A2 monooxygenases catalysing oxidation of IQ to *N*-hydroxy-IQ was effectively inhibited by 16 flavonoids. Flavones and flavonols are in general more potent enzyme inhibitors than flavanones, isoflavones and chalcones (Edenharder *et al*., 1997). The parent compounds of flavones and flavonols and a non-polar flavone, tangeretin, are the most potent inhibitors of MROD and EROD activites, but there is no clear relationship between an increasing number of hydroxyl groups and reduced inhibition of enzyme activity.

Flavonoids have no effect on the NADPH-dependent cytochrome P450 reductase activity (Edenharder *et al*., 1997). Antimutagenic acitivity of flavonoids against IQ induced mutagenesis has shown to not only involve inhibition of membrane bound cytochrome P450 dependent monooxygenases, but also various soluble enzymatic factors, which suggest interactions with

biological membranes and effects on expression and fixation of DNA damage (Edenharder *et al*., 1997). Inhibition of AFB_1 induced mutagenesis by flavonoids may be the result of interaction with components of the microsomal enzyme system, thus interfering with the bioactivation of AFB_1 (Francis *et al*., 1989).

G. Conclusion

A number of epidemiological studies during the last decade have linked tea consumption (especially green tea) to a reduced risk of cancer in humans, but the protective effect of tea has, however, not been demonstrated above all doubt (Blot *et al*., 1997; Dreosti *et al.,* 1997, Birt *et al.,* 2000). This is thought to be the consequence of tea not being the main objective in some of the epidemiological studies. As a result information on tea consumption is limited and often confused by multiple other lifestyle variables (Kohlmeier *et al*., 1997).

The effect of tea in animal models of carcinogenesis is unique in one respect: no other agent tested for possible chemoprevention effects in animal models has elicited such strong activity as tea and its components at the concentrations usually consumed by humans (Dreosti *et al.,* 1997). No data is currently available on the effect of rooibos and honeybush teas, since no epidemiological studies have been conducted. The effect of rooibos and honeybush teas in humans can, however, not be assumed to be similar to that of green and black teas, due to vast differences in composition.

The potential protective effects of teas demonstrated *in vitro* and from animal studies *in vivo* are convincing, and encompass several important mechanisms that suggest possible beneficial effects of tea (*Camellia sinensis*) and major phenolic constituents at most stages of cancer development (Yang & Wang, 1993; Katiyar & Mukhtar, 1996; Dreosti *et al.,* 1997*)*.

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Chapter 3

Antimutagenic activity of fermented and unfermented *C. intermedia, C. subternata, C. genistoides and C. sessiliflora* (honeybush) compared to fermented and unfermented *Aspalathus linearis* (rooibos) and *Camellia sinensis* (black, oolong and green) teas against aflatoxin B_1

Abstract

Antimutagenic activity of aqueous extracts prepared from four fermented and unfermented *Cyclopia spp.* (honeybush) was compared to that of fermented and unfermented *Aspalathus linearis* (rooibos) and *Camellia sinensis* (black, oolong and green) teas in the *Salmonella* mutagenicity assay with tester strain TA100 and aflatoxin B_1 (AFB_1) as mutagen. Composition of the teas was investigated by spectrophotometric analysis of the total polyphenol, flavanol and flavonol/flavone contents and HPLC analysis of the major phenolic constituents in rooibos and *Cyclopia spp.*, to elucidate their respective roles in antimutagenic potency of the teas. Black tea overall exhibited the highest protection against AFB_1 with fermented *C. intermedia* offering the least protection. Fermentation (oxidation step during tea processing) resulted in increased antimutagenic activity of *Camellia sinensis* and rooibos teas. The antimutagenic activity of *Cyclopia spp.* decreased with fermentation except for *C. genistoides*. The overall antimutagenic activity of the teas correlated moderately ($r = 0.551$, $P < 0.0001$) with the flavanol, and to a lesser extent, the total polyphenol content ($r = 0.436$, $P < 0.0001$). Separate correlations revealed that the antimutagenic activity of *Camellia sinensis* teas does not correlate with their content of respective phenolic groups. Correlations with the total polyphenol ($r = 0.805$, $P < 0.0001$) and flavanol content ($r = 0.653$, $P < 0.0001$) of *Cyclopia spp.* and the flavonol/flavone content ($r = 0.751$, $P = 0.005$) of rooibos tea with antimutagenic activity were observed. Antimutagenic activity of rooibos tea showed a strong negative correlation ($r = -0.918$, $P < 0.0001$) with its aspalathin content, while positive correlations for the flavones, orientin ($r = 0.674$, $P = 0.023$) and iso-orientin ($r = 0.728$, $P = 0.011$), were observed. Antimutagenicity of *Cyclopia spp.* correlated weakly ($r = 0.363$, $P = 0.012$) with its hesperidin content, while no correlation was observed with the mangiferin content. Flavonoid glycosylation appeared to influence antimutagenic acitivity against AFB_1, as the glycosides aspalathin, hesperidin and mangiferin had relatively weak antimutagenic activity compared to that of luteolin, chrysoeriol, hesperetin and (-) epigallo-catechin gallate (EGCG). Plant variation was shown to influence antimutagenic activity and

significant differences were observed in antimutagenic activity and phenolic composition of the different samples of fermented and unfermented *Cyclopia spp*. and rooibos tea tested in this study.

Introduction

Information regarding antimutagenic, anti-cancer and chemopreventive properties of the South African herbal teas, *Cyclopia spp.* (honeybush tea) and *Aspalathus linearis* (Burm. Fil) R. Dahlgr. spp. *linearis* (rooibos tea) is limited. The protective effect of fermented and unfermented honeybush tea (*C. intermedia*) has been demonstrated against metabolically activated AFB_1 and 2-acetylaminofluorene (2-AAF) with tester strains TA100 and TA98, respectively (Marnewick *et al*., 2000). Using tester strain TA98, Richards (2002) investigated several species of economic importance, including *C. intermedia*, and established that fermented and unfermented *C. intermedia, C. subternata* and *C. sessiliflora* protect against metabolically activated 2-AAF in the *Salmonella* mutagenicity assay, with *C. sessiliflora* exhibiting the highest protection against 2-AAF (Richards, 2002). Fermented *C. genistoides* demonstrated antimutagenic activity against 2-AAF-induced mutagenesis, while its unfermented counterpart enhanced mutagenicity. However, it did not exhibit a mutagenic response in the absence or presence of S9 (Richards, 2002). Fermentation resulted in decreased antimutagenic potency of *C. intermedia* and *C. sessiliflora* against 2-AAF, while the antimutagenic activity of *C. subternata* was not significantly affected (Richards, 2002).

Cyclopia spp. contain a complex mixture of phenolic compounds with qualitative (Ferreira *et al*., 1998; Kamara *et al*., 2003; Kamara *et al*., 2004) and quantitative differences between species (Richards, 2002, Joubert *et al*., 2003). Significant differences exist in the mangiferin and hesperidin content, two major phenolics present in *Cyclopia spp.,* in two types of *C. genistoides* (Overberg and West Coast types) (Joubert *et al*., 2003). Processing influences the phenolic composition of *Cyclopia spp.* significantly, resulting in markedly lower levels of mangiferin/isomangiferin (quantified together) and hesperidin in fermented *C. intermedia, C. subternata, C. genistoides* and *C. sessliflora,* compared to unfermented samples (Richards, 2002). Hesperetin and mangiferin exhibit an antimutagenic effect, while hesperidin enhances mutagenicity when using 2-AAF as a mutagen (Richards, 2002). Luteolin, a flavone that has been isolated from both *C. intermedia* (Ferreira *et al*., 1998) and *C. subternata* (Kamara *et al*.,

2004), has potent antimutagenic activity in the *Salmonella* mutagenicity assay (Samejima *et al*., 1995; Richards, 2002).

Rooibos tea, both fermented and unfermented, possesses antimutagenic activity against metabolically activated 2-AAF in the *Salmonella* mutagenicity assay with tester strain TA98 (Standley, 1999; Marnewick *et al*., 2000). Standley *et al.* (2001) demonstrated that processing results in reduced antimutagenic potency of rooibos tea against 2-AAF. Marnewick *et al*. (2000) found the same trend for protection against AFB_1-induced mutagenesis using tester strain TA100. Processing significantly influences the phenolic composition of rooibos (Joubert, 1996). A substantial decrease in the principle monomeric flavonoids of rooibos i.e. the dihydrochalcones, aspalathin and nothofagin, occurs during fermentation (Joubert, 1996).

Other phenolic compounds present in rooibos tea include the flavones, iso-orientin, orientin (Koeppen *et al*., 1962; Koeppen & Roux, 1965), vitexin, iso-vitexin, chrysoeriol (Rabe *et al*., 1994) and luteolin (Snyckers & Salemi, 1974), and the flavonols, rutin, isoquercitrin (Koeppen *et al*., 1962), quercetin (Snyckers & Salemi, 1974) and hyperoside (Bramati *et al*., 2003).

Aflatoxins are well-known foodborne carcinogens produced by *Aspergillus flavus,* recognised as one of the major risk factors in the development of liver cancer in human populations (Wogan, 1992; Wogan *et al*., 2004).

In the present study the protective effect of fermented and unfermented *Cyclopia spp.* was compared to that of fermented and unfermented rooibos tea, as well as *Camellia sinensis* teas (black, oolong and green teas) in the *Salmonella* mutagenicity assay using tester strain TA100 and AFB_1 as mutagen. The prevelance of mycotoxins in human foodstuffs was the main reason for selection of AFB_1 as model carcinogen in this study. Six samples obtained from different batches of plant material of each species were included to investigate the affect of natural plant variation on the antimutagenic potency of tea. The protective effect of selected phenolic compounds against AFB_1-induced mutagenesis was also assessed. These included aspalathin, the principle monomeric flavonoid in unfermented rooibos tea (Koeppen & Roux, 1966) and a major compound in fermented rooibos tea (Rabe *et al*., 1994; Joubert, 1996). Other selected phenolic compounds included the flavones, luteolin, present in fermented (Rabe *et al*., 1994) and unfermented rooibos (Bramati *et al*., 2003), fermented *C. intermedia* (Ferreira *et al*., 1998) and unfermented *C. subternata* (Kamara *et al*., 2004) and chrysoeriol, present in fermented (Rabe *et al*., 1994) and unfermented rooibos tea (Bramati *et al*., 2003). The flavanones tested included eriodictyol and hesperetin, present in fermented *C. intermedia* (Ferreira *et al*., 1998), as well as hesperidin that has been identified in fermented *C. intermedia* (Ferreira *et al*., 1998), unfermented

C. subternata (Kamara *et al*., 2004) and unfermented *C. genistoides* (Joubert *et al*., 2003). The xanthone, mangiferin, present in high levels in commercially important *Cyclopia spp.* (Richards, 2002; Joubert *et al*., 2003), was also included for antimutagenic testing. Antimutagenic activity of the most abundant catechin in green tea, (-) epigallocatechin gallate (EGCG), reported to be one of the major antimutagenic principles of green tea (Kada *et al*., 1985) and a phenolic constituent of unfermented *C. subternata* (Kamara *et al*., 2004), was determined as reference. Different phenolic groups as well as the individual phenolic compounds in aqueous extracts from fermented and unfermented *Cyclopia spp.* and fermented and unfermented rooibos tea were quantified to investigate their possible contribution to the antimutagenic activity of the respective teas.

Materials and Methods

Chemicals

Luteolin (> 90%), eriodictyol (HPLC grade) and chrysoeriol (HPLC grade) were purchased from Extrasynthèse (Genay, France). (-) Epigallocatechin gallate (EGCG) from green tea (> 95%), mangiferin (xanthone-*C*-glucoside) from *Mangifera indica* leaves, hesperidin (97%), hesperetin (≥ 95%), potassium chloride (KCl) (≥ 99.9%; ACS reagent), AFB_1, D-glucose-6-phosphate (disodium salt hydrate), β-nicotinamide adenine dinucleotide phosphate (β-NADP), methanol (100% HPLC-grade) and bovine serum albumin (BSA) were obtained from Sigma Chemicals Co. (St. Louis, USA). Di-sodium hydrogen orthophosphate (≥ 99.0; anhydrous Na_2HPO_4), sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$, general purpose reagent) and formic acid (98-100%) were purchased from BDH Chemicals Ltd. (Poole, England). Dimethyl sulfoxide (DMSO for UV-spectroscopy) (≥ 99.8%) was supplied by Fluka/Sigma-Aldrich Chemie (Steinheim, Germany). Aspalathin (≥ 95% as determined by HPLC and LC-MS) was isolated from unfermented rooibos tea at the PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis) unit of the Medical Research Council (MRC, Bellville, SA) (Snijman *et al*., unpublished data). Bacto agar was obtained from Difco Laboratories (Detroit, USA) and nutrient broth from Oxoid (Hampshire, UK). L - histidine was supplied by Merck (Darmstadt, Germany) and D-biotin was from ICN Biomedical Inc. (Ohio, USA). The genotype of the tester strain, *Salmonella typhimurium* TA 100, was obtained from Dr. B.N. Ames (Berkely University, California, USA) and Aroclor 1254 from Monsanto (USA). Purified water used for dissolving freeze-dried aqueous extracts for HPLC analysis refers to deionised and RO-treated water

(Modulab Water Purification System, Continental Water Systems Corporation, Separations (Cape Town, SA), further purified using a Milli-Q-Academic (Millipore) water purifier. Analytical grade solvents and chemicals were used except if stated otherwise.

Preparation of samples

Plant material

Six batches (*ca*. 5 kg per batch) of *C. intermedia* were harvested in the Haarlem area in March 2000. *Cyclopia subternata* (*ca*. 5 kg per batch) was harvested from 2- and 3-year-old plantations in Waboomskraal, Outeniqua and a 2-year-old plantation in Du Toitskloof (May 1997). Six batches of *C. genistoides* (*ca*. 4 kg per batch, West Coast type) were harvested from a 5-year-old plantation at Pearly Beach (March 2001). *Cyclopia sessiliflora* (*ca*. 4 kg, six batches) were obtained from the ARC experimental farm, Helderfontein, Stellenbosch (February 2001). Each batch comprised more than one plant.

Commercial samples of rooibos tea (three samples each of Super grade and Standard grade tea) were obtained from Rooibos Ltd, Clanwilliam and six samples of green, black and oolong teas each, were purchased from commercial retailers.

Preparation of tea extracts

Freeze-dried aqueous extracts from *Cyclopia spp.*, prepared by Richards (2002), were used in this study for the continued investigation of the antimutagenic activity of honeybush tea.

Freeze-dried extracts were prepared from rooibos, black, oolong and green teas, following the same method used by Richards (2002). The preparation procedure involved steeping 100 g of milled tea (1 mm sieve) in 1000 mL freshly boiled deionised water for 5 minutes. Extracts were then coarse filtered with a Buchner filter, using a 125 μm synthetic mesh cloth (Polymer PES D25/35 supplied by Swiss Silk Bolting Cloth Mfg. Co. Ltd, Zurich, Switzerland), followed by filtration with Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England) to remove finer particles. The filtrates were freeze-dried in an Atlas pilot-scale freeze-drier (Denmark model, Copenhagen, Denmark, 40ºC shelf temperature) after being frozen at -20ºC in plastic trays (170 x 115 x 30 mm). The freeze-dried aqueous extracts were placed in clear glass vials and stored in desiccators under vacuum in the dark.

Phenolic content of aqueous extracts

Yield of aqueous extracts from the plant material and total polyphenol, flavanol and flavonol/flavone contents of aqueous extracts

The yield of freeze-dried aqueous extracts from the plant material as well as the total polyphenol, flavanol and flavonol/flavone contents of the freeze-dried aqueous extracts, prepared from fermented and unfermented *C. intermedia, C. subternata, C. genistoides* and *C. sessiliflora*, were determined by Richards (2002). Analysis of the fermented and unfermented rooibos and black oolong and green tea extracts were carried out following the same experimental procedure as Richards (2002):

The total aqueous soluble solids, representative of the yield of freeze-dried aqueous extracts (hereafter referred to as aqueous extracts) from the plant material, was determined gravimetrically and expressed as g 100 g^{-1} of the plant material. The total polyphenol (Singleton & Rossi, 1965) and flavanol contents (McMurrough & McDowell, 1978) of the aqueous extracts were determined colorimetrically and expressed as g gallic acid equivalents 100 g^{-1} and g catechin equivalents 100 g^{-1} aqueous extract, respectively. The flavonol/flavone content of the aqueous extract was estimated spectrophotometrically at 360 nm (Mazza *et al*., 1999) and expressed as g quercetin equivalents 100 g^{-1} aqueous extract.

Individual phenolic content of aqueous extracts prepared from Cyclopia spp.

Quantification of phenolic compounds in aqueous extracts prepared from fermented and unfermented *Cyclopia spp.* (*C. intermedia, C. subternata, C. genistoides* and *C. sessliflora*) was conducted by reversed-phase HPLC, according to the method of Joubert *et al*. (2003). The HPLC system (Merck Hitachi, LaChrom system, Merck, Darmstadt, Germany) consisted of a solvent pump (LC-7100), autosampler (L-7200), UV detector (L-7450), diode array detector (DAD) (L-7450), interface (D-7000) and a D-7000 HPLC system manager version 4.1 for system control and data acquisition and analysis. Solvents were degassed in-line with a Phenomenex Degasser Model DG-4400 (Separations, Cape Town, SA). The Phenomenex RP/C_{18} 5µm Jour Guard column and Synergy Phenomenex MAX-RP C_{12} 80A column with TMS end-capping (C_{12} reverse-phase, 4µm; 150 x 4.6 mm ID) were purchased from Separations (Cape Town, SA).

Stock solutions of 10 mg mL^{-1} and 6 mg mL^{-1} were prepared from aqueous extracts of fermented and unfermented honeybush tea, respectively, by dissolving in purified water utilising ultra-sonication. The stock solutions were filtered through a 25 mm 0.45 µm Millipore Millex-

HV Hydrophilic PVDF syringe filter (Microsep, Bellville, SA) directly into a HPLC sample vial for injection (10 µL) in duplicate, without further dilution. Separations were carried out at 30˚C with the temperature maintained by a column oven. Tentative peak identity was determined by means of retention time and comparison to UV spectra of external HPLC standards. Quantification at 280 nm of compounds was based on peak area, obtained with valley-to-valley integration, using external standards. Quantities were expressed as a percentage of the aqueous extract. The concentration ranges of external standards were based on the expected levels of compounds in aqueous extracts and are given in Table 1.

Individual phenolic content of aqueous extracts prepared from rooibos tea

Quantification of phenolic compounds in aqueous extracts prepared from fermented and unfermented rooibos tea was conducted by reversed-phase HPLC, according to the method of Joubert (1996) with a slightly modified solvent program (Table 2). The HPLC system used was a Waters LC Module I Plus with a Waters 2996 Photodiode array detector (Microsep, Bellville, SA). Millennium 32 version 4 software was used for system control and data acquisition.

Separations were performed on a LiChrospher 100 RP-18 (C_{18} column, 5 µm particle size, 250 x 4 mm, ID) column with a LiChrospher 100 RP-18 (C_{18} column, 5 µm particle size) guard column, both purchased from Merck (Cape Town, SA). The mobile phase was degassed in-line with a Jour X-Act degasser (Separations, Cape Town, SA). Stock solutions (5 mg mL^{-1}) of the aqueous extracts of fermented and unfermented rooibos tea were prepared by dissolving in purified water and ultra-sonication. Stock solutions of the extracts of unfermented rooibos were diluted 50 times for aspalathin quantification and five times for quantification of the other compounds, before filtration. Stock solutions of extracts of fermented rooibos tea were filtered through a 25 mm 0.45 µm Nylon syringe filter directly into a HPLC sample vial for injection without further dilution. An injection volume of 20 µL was used and separations were carried out at 38˚C. Tentative identification of compounds was achieved with a photodiode array detector and comparison to retention time of the standards. Quantification of the dihydrochalcones, i.e. aspalathin and nothofagin at 288 nm, and the flavones and flavonols at 255 nm, was done in duplicate and based on peak area, obtained with valley-to-valley integration, using external standards. Quantities were expressed as a percentage of the aqueous extract. The concentration ranges used for quantification of compounds were based on their expected concentration levels in the aqueous extracts and are summarised in Table 3.

Table 1 Concentration ranges, calibration curves and linearity (R^2) of external standards used for HPLC quantification of phenolic compounds in aqueous extracts from *Cyclopia spp.*

Compound type	Compound[a]	Concentration range (µg injected)	Calibration curve	R^2
Xanthone	Mangiferin[b]	0.050-18.350	y = 1031603x + 64729.17	0.999
	Isomangiferin[c] (mangiferin)	0.050-18.350	y = 1031603x + 64729.17	0.999
Flavone	Luteolin[d]	0.002-0.090	y = 2187016x - 2187016	0.997
Flavanone	Eriocitrin[e]	0.008-3.340	y = 1071925x + 7761.28	0.999
	Eriodictyol[d]	0.020-4.120	y = 1999013x - 6811.68	0.999
	Hesperidin[b] (97%)	0.016-6.360	y = 131642x + 4297.06	0.999
	Hesperetin[e]	0.005-2.000	y = 2452335x + 4371.67	0.999
	Naringenin[e]	0.020-1.840	y = 4616511x - 100456.20	0.997
	Narirutin[e]	0.011-4.220	y = 1196646x + 12649.14	0.999
Isoflavones	Formononetin[e]	0.020-3.140	y = 2489117x - 16969.07	0.998
Catechin	(-)Epigallocatechin gallate[b] (≥95%)	0.020-3.900	y = 745970x - 17157.97	0.991

[a] All compounds used were HPLC grade except for mangiferin.
[b] Obtained from Sigma Chemicals Co. (St Louis, USA).
[c] Isomangiferin quantified as mangiferin equivalents.
[d] Obtained from Carl Roth (Karlsruhe, Germany).
[e] Obtained from Extrasynthèse (Genay, France).

Table 2 Modified solvent gradient program for separation of phenolic compounds of rooibos tea by reversed-phase HPLC with 2% (v/v) formic acid solution (diluted with purified water) as solvent A and 100% methanol as solvent B. The last 15 minutes were used for equilibration.

Time (min)	Flowrate (mL/min^{-1})	% A (2% Formic acid)	% B (100% methanol)
0	0.40	70	30
15	0.40	65	35
25	0.40	60	40
35	0.40	50	50
40	0.40	40	60
50	0.40	20	80
60	0.40	40	60
80	1.00	70	30
95	1.00	70	30

Table 3 Concentration ranges, calibration curves and linearity (R^2) for external standards used for HPLC quantification of phenolic compounds in aqueous extracts from rooibos tea.

Compound type	Compound[a]	Concentration range (µg injected)	Calibration curves	R^2
Dihydrochalcone	Aspalathin[b] (≥ 95%)	0.080-0.430	y = 4015115x + 34758.50	0.999
	Nothofagin[b] (≥ 95%)	0.080-0.400	y = 5656459x – 29060.75	0.994
Flavone	Chrysoeriol[c]	0.005-0.028	y = 8060389x – 4012.95	0.995
	Iso-orientin[c]	0.080-0.400	y = 5308099x + 49200.19	0.999
	Isovitexin[c]	0.086-0.430	y = 4382904x – 36049.95	0.994
	Luteolin[c]	0.003-0.013	y = 11177640x – 3290.00	0.994
	Orientin[d]	0.088-0.440	y = 5412852x + 26124.14	0.999
	Vitexin[c]	0.078-0.390	y = 6740934x – 3272.04	0.999
Flavonol	Quercetin[c]	0.009-0.043	y = 16058205x – 14694.35	0.995
	Quercetin (Isoquercitrin/rutin)[e]	0.243-1.216	y = 16058205x – 14694.35	0.995

[a] All compounds were of HPLC-grade.

[b] Compounds isolated from unfermented rooibos tea at the PROMEC unit, MRC, SA (Snijman *et al*., unpublished data) with purity determined by HPLC and LC-MS.

[c] Obtained from Extrasynthèse (Genay, France).

[d] Obtained from Roth (Karlsruhe, Germany).

[e] Isoquercitrin and rutin were quantified as quercetin equivalents (quantified together due to co-elution).

Antimutagenic activity in the Salmonella mutagenicity assay

Confirmation of genotype of tester strain TA 100

The genotype of the tester strain, *Salmonella typhimurium* TA 100, was confirmed according to the procedure described in Maron & Ames (1983), with five assay repeats of each test.

Confirmation of histidine requirement (His^-) of the tester strain involved circling two areas at the bottom of a plate containing minimal glucose (MG) (Vogel-Bonner medium E) agar. A drop of 0.5 mM biotin was placed in both areas, while a drop of 0.1 mM L-histidine was only added to one of the areas. Bacterial culture of TA100, prepared in nutrient broth and incubated overnight, was added to both areas and mixed properly and the plates were incubated for 12 hrs at 37ºC.

The presence of the *rfa* mutation in the tester strain was confirmed by testing for sensitivity to crystal violet. Overnight culture (0.1 mL) was added to 2.5 mL melted top agar, kept at 45˚C, and vortexed for 3 seconds before being poured and evenly distributed onto a MG plate. A 1 mg mL^{-1} (0.1%) crystal violet solution was prepared and 10 µL was transferred onto a sterile filter paper disc (1/4 inch), which was then positioned in the center of the MG plate.

The test for the *uvrB-bio* deletion mutation involved streaking the overnight culture across a nutrient agar plate in parallel stripes and then irradiating with a 15-W germicidal lamp at a distance of 33 cm for 8 seconds, with half of the plate covered. The irradiated plates were incubated for 12 hrs at 37ºC.

The presence of plasmid pKM101, referred to as the R-factor, was confirmed by application of commercial 10 µg of ampicillin discs (BBL sensi-disc, Becton Dickinson Microbiology Systems, Cockeysville, MD 21030, USA) to a plate seeded with the bacteria in the top layer as described above. The amipicillin disc was placed in the center of the plate and then incubated for 12 hrs at 37ºC.

Preparation of the liver homogenate (S9) fraction and the S9 mixture

Induction with Aroclor 1254 is recommended for liver homogenates used in testing for general mutagenesis (Maron & Ames, 1983). The induction procedure described by Czygan *et al.* (1973) included diluting Aroclor 1254 in sunflower oil to a concentration of 200 mg mL^{-1} and a single injection of 500 mg kg^{-1} to each male Fischer rat (± 200 g) five days before sacrifice.

Liver homogenate fractions were prepared according to the method of Maron & Ames (1983). Freshly excised livers were weighed and washed several times in chilled KCl before being homogenised in 0.15 M KCl (3:1 m/v) for 1 minute, using a Thomas homogeniser. The homogenate was filtered through double-layered cheesecloth and homogenised using 10 strokes

in a glass tissue-grinder with a tight plunger. The homogenate was centrifuged at 9000 *x g* for 10 minutes and aliquots of the supernatant (S9 fraction) were stored in glass vials at –80ºC until used.

The S9 fraction was thawed at room temperature and kept on ice for preparation of the S9 mixture. The protein concentration was determined according to the method described by Kaushal & Barnes (1986) with bovine serum albumin as standard. The cytochrome P450 content was assessed using the method of Omura & Sato (1964). The S9 mixture (50 mL) contained 2 mL of the S9 fraction; 1 mL $MgCl_2$-KCL salts (0.4 M $MgCl_2$, 1.65 M KCL); 0.25 mL 1 M glucose-6-phosphate; 2 mL 0.1 M NADP; 25 mL 0.2 M phosphate buffer (pH 7.4); and 19.75 mL sterile distilled water. The mixture was freshly prepared on the day of the assay and kept on ice while conducting the mutagenicity assay.

Stock solutions of 0.1 M NADP and 1 M glucose-6-phosphate were prepared with sterile distilled water and stored at -20ºC and thawed at room temperature on the day of the assay. The salt solutions and phosphate buffer were prepared with sterile distilled water, autoclaved and kept refrigerated. All the procedures were carried out under sterile conditions.

Assessment of optimal mutagen concentration to be used in the standard plate incorporation assay

The number of revertants induced by different concentrations of AFB_1 in the *Salmonella* mutagenicity assay, was determined according to the standard plate incorporation assay (Maron & Ames, 1983), in the presence of S9 to effect the metabolic activation. The optimum mutagen concentration was determined by performing the assay at concentrations of 10, 20 and 40 ng per plate.

Antimutagenicity of aqueous tea extracts and selected phenolic compounds

Antimutagenic activity of aqueous extracts prepared from fermented and unfermented *C. intermedia*, *C. subternata*, *C. genistoides, C. sessiliflora*, rooibos and *Camellia sinensis* teas (black, oolong and green) against AFB_1 was determined according to the standard plate incorporation assay (Maron & Ames, 1983), with some modification as illustrated in Fig. 1. Antimutagenicity was assessed by adding 0.1 mL of the mutagen (20 ng of AFB_1 per plate), 0.1 mL of the different tea extracts [0.5%, 1% and 2% (w/v)], 0.5 mL S9 mixture and 0.1 mL of an overnight bacterial culture per plate to 2 mL of molten top agar (containing 0.05 mM biotin-histidine) and then dispersed onto minimal glucose agar (Vogel-Bonner medium E) plates.

The antimutagenic properties of fermented and unfermented rooibos and *Camellia sinensis* teas were also determined at 0.1 % (w/v). A stock solution of AFB_1 was freshly prepared on the day of the assay using dimethyl sulfoxide (DMSO) as solvent. The aqueous extracts were dissolved in sterile water, diluted to the required concentration and filtered with a 0.45 μm filter, followed by a 0.22 μm filter (sterile Nylon Cameo filters, Separations, Cape Town, SA). Antimutagenic activity of the selected phenolic compounds (dissolved in DMSO) of the respective teas was determined at concentration levels of 0.1 mM and 0.3 mM per plate and included aspalathin, luteolin, chrysoeriol, eriodictyol, hesperidin, hesperetin, mangiferin and EGCG.

Mammalian liver S9 (0.7 nM cytochrome P450 mg^{-1} protein) in the S9 mixture was introduced at a level of 2 mg mL^{-1}. Control treatments (negative control) included TA100 in the presence of the S9 mixture to determine the spontaneous revertants, while positive control plates contained S9 mixture, TA100 and AFB_1 to determine the maximum number of revertants. The percentage inhibition values were calculated with the following formula:

$$\text{Inhibition (\%)} = 1 - \left[\frac{\text{Number of His}^+ \text{ revertants in the presence of tea extract/phenolic compound}}{\text{Number of His}^+ \text{ revertants in the absence of tea extract/phenolic compound}}\right]$$

The number of spontaneous revertants, determined in the absence of aqueous extracts/phenolic compounds and AFB_1, was subtracted from the numerator and denominator (Chen & Yen, 1997).

Statistical analysis

One-way analysis of variance (ANOVA) was performed with SAS version 8.2 to determine whether the composition in terms of the yield of aqueous extract and total polyphenol, flavanol, flavonol/flavone (*Cyclopia spp.*, rooibos and *Camellia sinensis*) and individual flavonoid content (*Cyclopia spp.* and rooibos) of teas differ. Shapiro-Wilk's test was used to test for non-normality and the Student's t-LSD (Least Significant Differences) was calculated at a 5% level ($P < 0.05$) to compare means.

Statistical analyses of data obtained with the *Salmonella* mutagenicity assay was performed with SAS version 9.1 and included tests for normality among the groups with the Kolmogorov Smirnof Test and testing for homogeneity of group variances with Levene's Test. A one-way analysis of variance (ANOVA) was performed for testing significant group differences followed by a post-HOC Tukey's Studentised Range test, to determine which groups differed significantly.

Where sample sizes were unequal, the Tukey-Cramér adjustments were made automatically. Means were correlated with the Pearson's correlation coefficient. An outlier was removed from the compositional data of rooibos tea for the correlations with antimutagenic activity. Statistical significance was considered at 5% ($P < 0.05$).

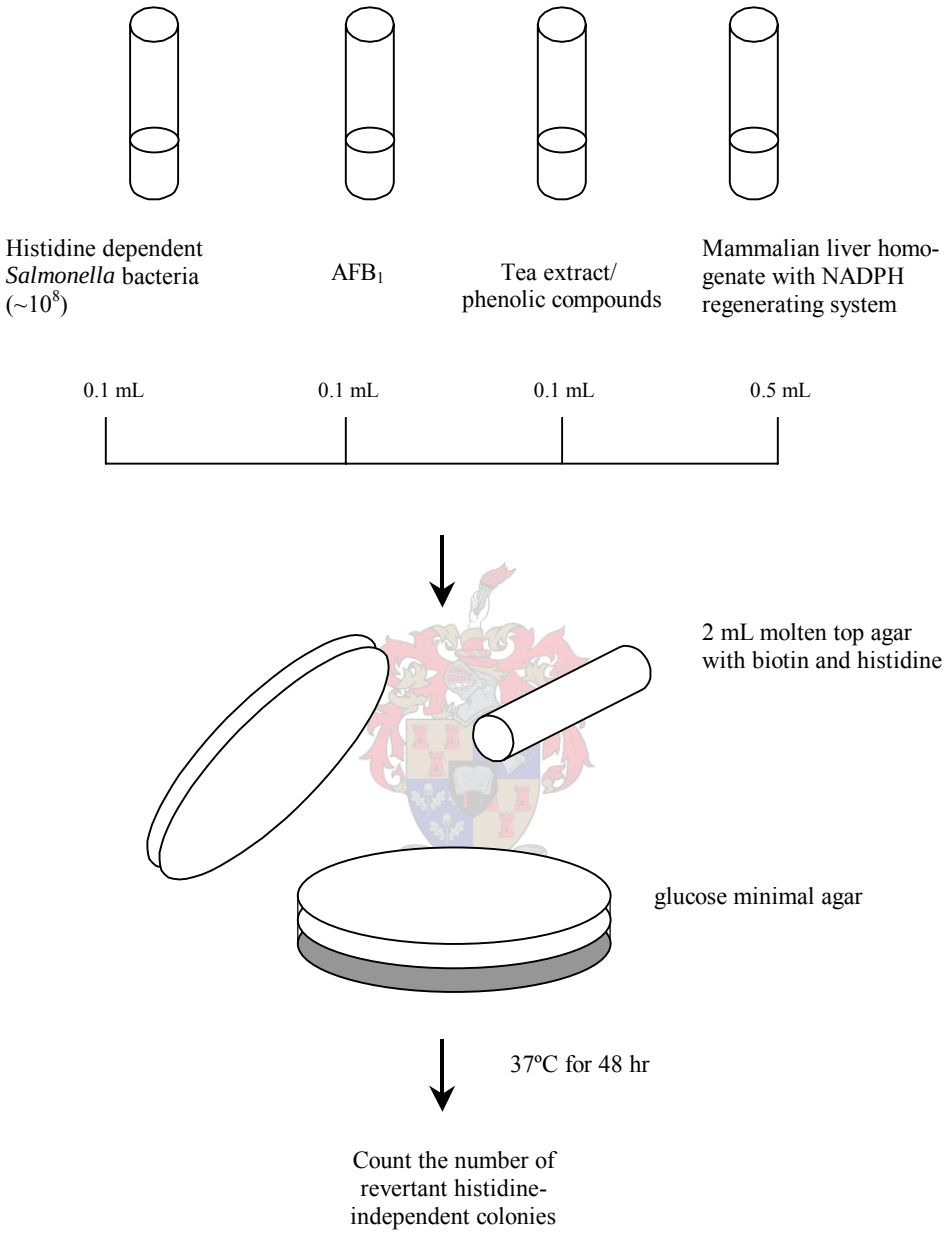


Figure 1 A diagrammatic representation of the steps followed to determine the antimutagenic activity of the aqueous tea extracts and individual phenolic compounds.

Results

Phenolic content of aqueous extracts

Yield of aqueous extract from the plant material and total polyphenol, flavanol and flavonol/flavone contents of aqueous extracts

The yield of aqueous extract from the plant material and the total polyphenol, flavanol and flavonol/flavone contents of the aqueous extracts of the teas differed significantly (Table 4). Fermentation significantly ($P < 0.05$) reduced the yield of aqueous extract and its total polyphenol and flavanol content. However, the effect of fermentation was less marked for *C. genistoides* than for other *Cyclopia spp.* with approximately 92% and 77% of the yield and total polyphenol content retained, respectively. The most significant ($P < 0.05$) reduction with fermentation was observed for *C. subternata,* with the yield reduced with *ca.* 41% and total polyphenol content with 46%. For plant material of the *Cyclopia spp.,* unfermented *C. genistoides, C. subternata* and fermented *C. genistoides* demonstrated the highest yield of aqueous extract and fermented *C. intermedia* the lowest. Unfermented *C. subternata* exhibited the highest total polyphenol and unfermented *C. intermedia* the highest flavanol content of the *Cyclopia spp.*, while flavonol/flavones were more abundant in the aqueous extracts of unfermented *C. genistoides,* followed by fermented *C. genistoides*, which was significantly ($P < 0.05$) higher than that of the other unfermented *Cyclopia spp.*

Fermentation significantly ($P < 0.05$) reduced the yield of aqueous extract from rooibos tea (*ca.* 39%), as well as the total polyphenol and flavanol content. The total polyphenol content of aqueous extracts from unfermented rooibos tea was significantly ($P < 0.05$) higher than for the other teas, including its fermented counterpart. However, fermentation resulted in a significantly ($P < 0.05$) higher flavonol/flavone content in fermented rooibos samples (12.12%) compared to unfermented samples (6.82%).

A decrease in the order green > black > oolong tea was evident for *Camellia sinensis* teas in terms of yield, total polyphenol and flavanol contents of the aqueous extracts, demonstrating a more substantial reduction with fermentation for oolong tea than for black tea. The flavonol/flavone content of *Camellia sinensis* teas decreased in the order black ≥ oolong > green tea, which is the reverse of the trend observed for the yield, total polyphenols and flavonol/flavones.

Table 4 Yields of aqueous extract from the plant material and phenolic content of aqueous extracts of fermented and unfermented *Cyclopia spp.*, fermented and unfermented rooibos and *Camellia sinensis* teas.

Tea[a]	Yields of aqueous extract[bc]	Total Polyphenols[bd]	Flavanols[be]	Flavonol/Flavones[bf]
C. intermedia[g] Fermented	16.96± 0.79 h	16.41± 1.50 e	0.61± 0.16 h	2.69± 0.37 h
C. intermedia[g] Unfermented	27.34± 1.49 f	30.23± 0.52 c	2.34± 0.14 d	3.34± 0.38 fg
C. subternata[h] Fermented	22.06± 1.63 g	17.49± 1.54 e	0.18± 0.03 j	2.86± 0.18 gh
C. subternata[g] Unfermented	37.16± 4.68 bc	32.24± 0.21 b	1.28± 0.17 e	3.50± 0.53 f
C. genistoides[g] Fermented	35.63± 1.20 cd	22.04± 1.02 d	0.41± 0.03 i	7.63± 0.54 c
C. genistoides[g] Unfermented	38.73± 0.42 ab	28.66± 1.24 c	0.86± 0.11 f	8.34± 0.60 b
C. sessiliflora[g] Fermented	22.48± 0.86 g	17.31± 1.51 e	0.14± 0.03 j	4.50± 0.35 e
C. sessiliflora[g] Unfermented	33.39± 1.74 e	29.86± 1.17 c	0.76± 0.05 fg	6.61± 0.29 d
Rooibos[g] Fermented	17.26± 2.90 h	29.26± 1.16 c	0.70± 0.04 gh	12.12± 0.34 a
Rooibos[g] Unfermented	28.40± 2.24 f	35.29± 1.80 a	1.23± 0.18 e	6.82± 0.94 d
Black tea[g] Fermented	33.62± 0.46 de	28.47± 1.49 c	6.28± 0.11 b	3.66± 0.16 f
Oolong tea[h] Semi-fermented	28.10± 1.29 f	23.03± 1.48 d	4.48± 0.05 c	3.39± 0.09 f
Green tea[h] unfermented	39.90± 1.27 a	33.36± 2.30 b	14.13± 0.85 a	2.70± 0.25 h

[a] Determinations for fermented and unfermented *Cyclopia spp*. were conducted by Richards (2002).

[b] Values within the same column followed by different letters differed significantly ($P < 0.05$).

[c] Yields of aqeous extracts are expressed as g 100 g^{-1} plant material.

[d] Total polyphenol content are expressed as g gallic acid equivalents 100 g^{-1} aqueous extract.

[e] Flavanol content is expressed as g catechin equivalents 100 g^{-1} aqueous extract.

[f] Flavonol/flavone content is expressed as g quercetin equivalents 100 g^{-1} aqueous extract.

[g] Values represent the means ± SD of six samples (n = 6) determined in triplicate.

[h] Values represent the means ± SD of five samples (n = 5) determined in triplicate.

The yield for *Camellia sinensis* teas were similar (P > 0.05) to that of unfermented *C. genistoides, C. subternata* and fermented *C. genistoides,* while fermented *C. intermedia* and fermented rooibos exhibited the lowest yield of aqueous extract.

Unfermented rooibos had the highest total polyphenol content of the teas, while fermented rooibos exhibited the highest flavonol/flavone content by far. The flavanol content of *Camellia sinensis* teas was significantly (P < 0.05) higher than in rooibos and *Cyclopia spp.*

Individual phenolic content of aqueous extracts prepared from honeybush tea

The average contents of individual phenolic compounds of the aqueous extracts from *Cyclopia spp.* are summarised in Table 5. Mangiferin was present in the highest concentration in unfermented *C. genistoides* (10.04%), while relatively high levels of this compound were also detected in fermented *C. genistoides* (4.29%), unfermented *C. sessiliflora* (4.39%) and unfermented *C. intermedia* (2.49%). The mangiferin content of fermented *C. subternata* (0.07%) was significantly (P < 0.05) lower than for other *Cyclopia spp.* Isomangiferin was not detected in most samples, while traces were present in fermented *C. subternata*, *C. genistoides* and *C. sessiliflora*. Luteolin was present at very low levels in *C. subternata*, *C. genistoides* and unfermented *C. sessiliflora*.

Of the flavanones, hesperidin was the major compound present, with the highest levels detected in unfermented *C. intermedia* (1.12%) and *C. genistoides* (0.92%). The hesperidin content of fermented *C. intermedia* and *C. genistoides* and fermented and unfermented *C. subternata* and *C. sessiliflora* varied between 0.27% and 0.50%. Other flavanones quantified were eriocitrin, present in fermented *C. subternata* and *C. sessiliflora* and unfermented *C. subternata*, *C. genistoides* and *C. sessiliflora*, and narirutin present at very low levels in all the species, except *C. genistoides*. Hesperetin was most ubundant in fermented *C. intermedia*, while low levels were present in unfermented *C. intermedia* and fermented *C. genistoides* and only traces in some of the samples of unfermented *C. genistoides* and *C. sessiliflora* were present. Naringenin and formononetin could not be detected in any of the samples, while trace amounts of eriodictyol were present in some of the samples.

Fermentation influenced the phenolic composition of the *Cyclopia spp.* (Table 5). The total flavanones were markedly higher in extracts from unfermented samples than in fermented counterparts. Fermentation resulted in significantly (P < 0.05) lower levels of hesperidin, except for *C. sessiliflora*. Eriocitrin levels in *C. subternata*, *C. genistoides* and *C. sessiliflora* and narirutin in *C. intermedia* and *C. sessiliflora* were significantly (P < 0.05) reduced by

Table 5 Quantitative data of individual flavonoids of aqueous extracts prepared from fermented and unfermented *Cyclopia spp.* as determined with reversed-phase HPLC analysis carried out on a Synergy MAX-RP C_{12} column with acetonitrile and 2% acetic acid in water as eluents.

Compound Type	Compound	*C. intermedia*		*C. subternata*		*C. genistoides*		*C. sessiliflora*	
		Fermented[a]	Unfermented[a]	Fermented[a]	Unfermented[a]	Fermented[a]	Unfermented[a]	Fermented[a]	Unfermented[a]
Xanthone	Mangiferin	0.23± 0.04 e	2.49± 0.57 c	0.07± 0.02 f	1.19± 0.38 d	4.29± 1.14 b	10.04± 0.75 a	0.19± 0.04 e	4.39± 0.34 b
	Isomangiferin	nd[b]	nd	trace/nd[c]	nd	trace	nd	trace	nd
Flavone	Luteolin	nd	nd	< 0.01/nd[d]	< 0.01	≤ 0.012	≤ 0.016	nd	< 0.01
Flavanone	Eriocitrin	nd	nd	0.26± 0.07[e] c	0.47± 0.09[e] a	nd	0.15± 0.02[e] d	0.13± 0.04[e] d	0.33± 0.06[e] b
	Eriodictyol	nd	nd	trace/nd	nd	nd	trace/nd	trace	trace
	Hesperidin	0.45± 0.07 cd	1.12± 0.05 a	0.27± 0.08 e	0.42± 0.17 cd	0.47± 0.01 cd	0.92± 0.08 b	0.45± 0.03 cd	0.50± 0.05 c
	Hesperetin	0.07± 0.02	≤ 0.02[e]	nd	nd	≤ 0.01[e]	trace/nd	trace/nd	nd
	Naringenin	nd	nd	nd	nd	nd	nd	nd	nd
	Narirutin	0.02± 0.004[e] c	0.04± 0.020[e] a	0.04± 0.050[e] a	0.04± 0.010[e] a	nd	nd	0.02± 0.004[e] c	0.03± 0.003[e] b
Isoflavone	Formononetin	nd	nd	nd	nd	nd	nd	nd	nd
	Total flavanones[f]	0.53± 0.007	1.33± 0.06	0.58± 0.07	0.93± 0.15	0.67± 0.06	1.07± 0.09	0.59± 0.04	0.85± 0.06

[a] Values (g 100 g^{-1} aqueous extract) represent the means ± SD of six samples (n = 6) determined in duplicate. Means in the same row followed by different letters differed significantly ($P < 0.05$).

[b] Not detected.

[c] Trace amounts present in some of the samples, while not detected in others (see Addendum A, Table 1).

[d] Compound levels were less than 0.01 in some of the samples, while not detected in others (see Addendum A, Table 1).

[e] Levels too low to effectively verify with DAD, therefore identification based on retention time.

[f] The total flavanone content calculated as the sum of hesperidin, hesperetin, eriocitrin and narirutin contents.

fermentation. The xanthone content was also affected by fermentation, with the mangiferin content significantly ($P < 0.05$) lower in the fermented C*yclopia spp.* The mangiferin content of *C. genistoides* was reduced by 57% by fermentation, while reduction in other species comprised more than 90%.

Typical chromatograms of the external standards used for quantification and of fermented and unfermented samples of the four species analysed, are presented in Addendum A, Figs. 1-3.

Apart from the differences in the phenolic profiles of the respective *Cyclopia spp.*, considerable amounts of an unidentified compound (RT = 19.63 min) was present in *C. intermedia*. Relatively large quantities of two unidentified compounds were present in *C. subternata* at elution times of 12.42 and 12.72 min, while the second compound also appeared to be present in *C. sessiliflora*. The levels of these compounds decreased with fermentation (Figs. 2 and 3, Addendum A). Considerable variation in the levels of compounds in the individual samples of different species was evident. The data is presented in Addendum A, Table 1.

Individual flavonoid content of aqueous extracts prepared from rooibos tea

The individual flavonoid content of the aqueous extracts of fermented and unfermented rooibos tea is summarised in Table 6. The major compounds were aspalathin in unfermented rooibos and iso-orientin, orientin and aspalathin in fermented samples. Fermentation resulted in more than 90% reduction in aspalathin, while orientin and iso-orientin increased by 17.14% and 24.42% respectively with fermentation. The levels of vitexin did not differ significantly ($P > 0.05$) in fermented and unfermented samples.

The aspalathin content of unfermented samples (6.77%) was significantly ($P < 0.05$) higher than that of orientin (0.58%) and iso-orientin (0.65%), but in fermented tea iso-orientin was the major compound (0.86%), followed by orientin (0.70%) and aspalathin (0.57%). Nothofagin was present in very low quantities in fermented and unfermented samples, but could not be quantified.

Isoquercitrin and rutin were quantified together as quercetin equivalents, due to co-elution. Very low levels of isovitexin, isoquercitrin/rutin and quercetin were detected in unfermented samples. The isovitexin and isoquercitrin/rutin levels in fermented samples were similar, but significantly ($P < 0.05$) higher than that of quercetin. The total flavones comprised 1.86% of the aqueous extracts of fermented rooibos, compared to 1.29% for unfermented rooibos. The total flavonoids in unfermented samples (8.06%) were more than double of that in fermented samples.

Table 6 Quantification of the individual flavonoids in aqueous extracts of fermented and unfermented rooibos tea as determined with HPLC on a reversed-phase LiChrospher 100 RP–18 C_{18} column with 2% formic acid in water and 100% methanol as eluents.

Compound type	Compound	Fermented[a]	Unfermented[a]
Dihydrochalcone	Aspalathin	0.57± 0.17*[b] c	6.77± 1.83* a
	Nothofagin	< 0.02[c]	< 0.02
Flavone	Chrysoeriol	nd[d]	nd
	Iso-orientin	0.86± 0.07* a	0.65± 0.11* b
	Isovitexin	0.23± 0.02 d	< 0.02
	Luteolin	nd	nd
	Orientin	0.70± 0.05* b	0.58± 0.10* b
	Vitexin	0.10± 0.01 e	0.06± 0.01 c
	Isovitexin	0.23± 0.02 d	< 0.02
Flavonol	Isoquercitrin/rutin	0.30± 0.04 d	< 0.02
	Quercetin	0.02± 0.00 f	< 0.02
	Total flavones[e]	1.86± 0.13	1.29± 0.22
	Total flavonoids[f]	2.73± 0.21	8.06± 1.85

[a] Values (g 100 g^{-1} aqueous extract) represent the means ± SD of six samples (n = 6) determined with duplicate analysis.

[b] Means marked with a symbol (*) indicate that the level of the compound in fermented samples differed significantly ($P < 0.05$) from that in the unfermented samples. Means in the same row followed by different letters differed significantly ($P < 0.05$).

[c] Compounds could not be quantified, but trace amounts were detected.

[d] Not detected.

[e] Means represent the sum of orientin, iso-orientin, isovitexin and vitexin contents.

[f] Means represent the sum of all the flavonoids quantified.

Typical chromatograms of the external standards used for quantification of fermented and unfermented rooibos tea samples are presented in Addendum A, Figs. 4 and 5. Variation in the flavonoid content of individual samples of fermented and unfermented rooibos is illustrated by the data for individual samples presented in Addendum A, Table 2. The most variation was evident for the aspalathin content of unfermented rooibos, ranging between 3.49 and 8.29%.

Antimutagenic activity in the Salmonella mutagenicity assay

Confirmation of genotype of tester strain TA100

Histidine requirement (His^- character) of the tester strain was confirmed by visible growth on the area containing both L-histidine and biotin and no growth on the area with only biotin (Fig. 2a). Increased permeability of the bacteria to large molecules due to the *rfa* mutation was found to be present in the tester strain, since a zone of inhibition (about 14 mm) was present after 12 hrs of incubation (Fig. 2b). UV sensitivity of the tester strain was evident after incubation for 12 hrs at 37ºC, since growth was only visible on the un-irradiated side of the plate (Fig. 2c). Absence of zones of inhibition around the ampicillin-containing disc in Fig. 2d indicated the presence of the plasmid pKM101 in the tester strain used.

Assessment of optimal mutagen concentration to be used in the standard plate incorporation assay

An increase in the concentration of AFB_1 resulted in an increased number of revertants in the *Salmonella* mutagenicity assay. Concentrations of 10, 20 and 40 ng per plate induced 265, 441 and 578 revertant colonies per plate, respectively. A concentration of 20 ng per plate was selected for antimutagenicity testing, showing to be the optimal concentration for antimutagenic testing, as it produced an adequate number of revertants above the spontaneous revertants (120 revertants per plate) to assess antimutagenic activity of the different teas and phenolic compounds.

Antimutagenic activity of aqueous tea extracts and selected phenolic compounds

Inhibition of metabolically activated AFB_1-induced mutagenesis by the tea at concentrations of 0.1, 0.5, 1 and 2 mg aqueous extract per plate is summarised in Table 7.

A significant ($P < 0.05$) dose response effect was evident for the teas, except for black and oolong teas, which nearly completely inhibited mutagenesis at 0.5, 1 and 2 mg aqueous extract per plate and 1 and 2 mg per plate, respectively. However, at a concentration level of 0.5 mg

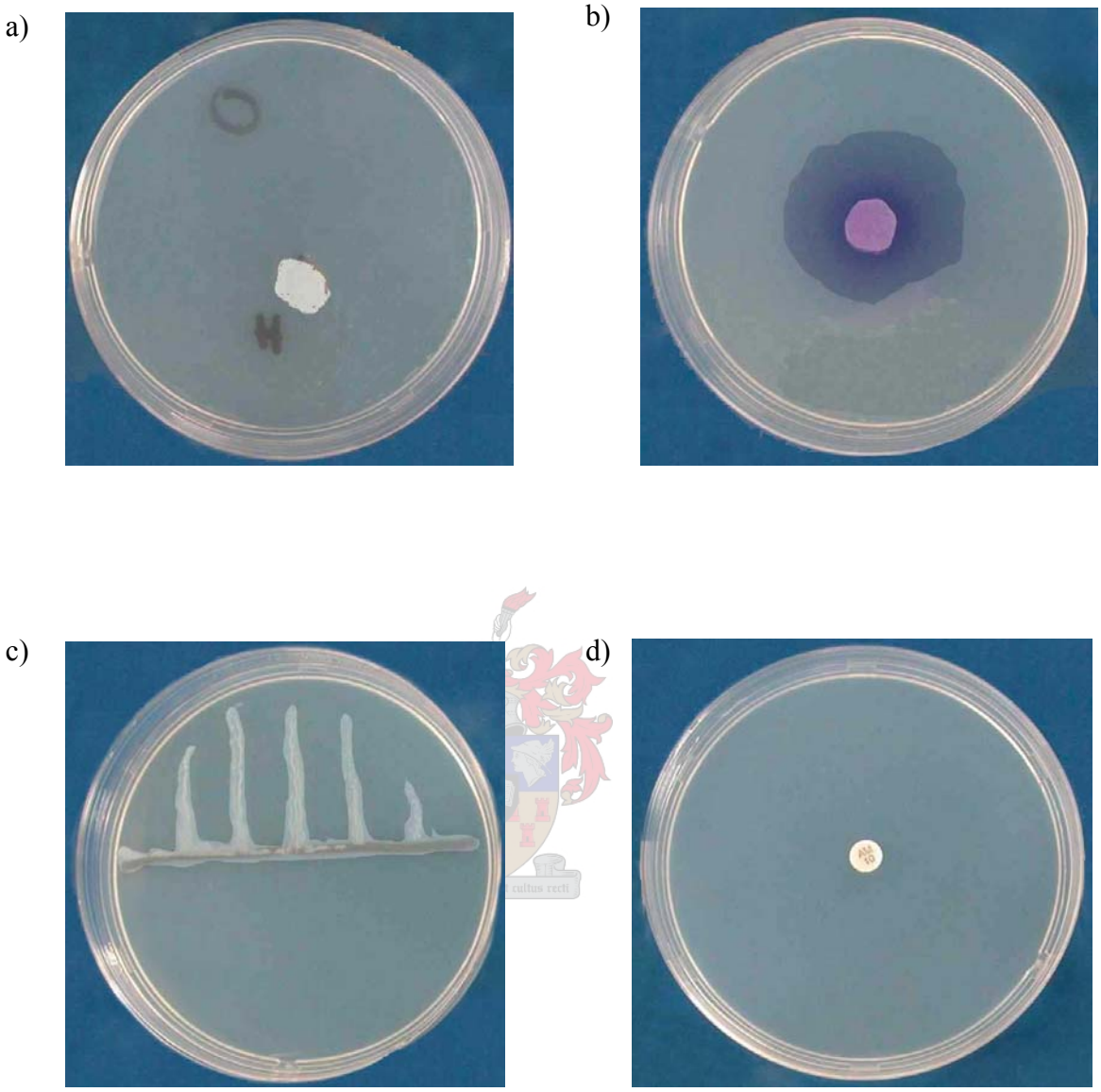


Figure 2 Confirmation of the genotype of tester strain TA100 according to the method described in Maron & Ames (1983) (a) histidine requirement, (b) *rfa* mutation, (c) *uvrB* mutation and (d) R-factor.

Table 7 Protective effect of aqueous extracts prepared from fermented and unfermented *Cyclopia spp.*, fermented and unfermented rooibos, black, oolong and green teas against AFB_1 (20 ng per plate) induced mutagenesis in *Salmonella typhimurium* TA100.

Tea	0.1 mg[ab]	0.5 mg[ac]	1 mg[ac]	2 mg[ac]
C. intermedia Fermented		25.83 ± 12.43[d] k*[e] (314 ± 44)	50.18 ± 12.85 j** (249 ± 45)	79.21 ± 8.14 j[#] (171 ± 23)
C. intermedia Unfermented		77.28 ± 7.32 de* (177 ± 25)	93.15 ± 6.43 bc** (136 ± 23)	97.28 ± 4.81abcdef[#] (124 ± 17)
C. subternata Fermented		39.53 ± 12.56 j* (277 ± 39)	60.77 ± 12.29 i** (220 ± 38)	78.68 ± 12.48 j[#] (171 ± 33)
C. subternata Unfermented		73.66 ± 10.97 ef* (186 ± 38)	90.48 ± 6.66 cde** (143 ± 25)	96.54 ± 4.75 abcdefg[#] (127 ± 21)
C. genistoides Fermented		47.05 ± 13.54 hi* (257 ± 41)	71.12 ± 7.57 g** (193 ± 27)	87.87 ± 7.02 h[#] (149 ± 22)
C. genistoides Unfermented		49.79 ± 9.8 h* (248 ± 26)	67.08 ± 13.4 gh** (203 ± 35)	83.24 ± 9.96 ij[#] (160 ± 25)
C. sessiliflora Fermented		42.23 ± 15.14 ij* (286 ± 37)	65.78 ± 7.15 hi** (207 ± 20)	85.61 ± 8.56 hi[#] (154 ± 22)
C. sessiliflora Unfermented		71.79 ± 11.28 f* (193 ± 38)	90.92 ± 8.02 bcde** (142 ± 25)	98.58 ± 5.06 abc[#] (121 ± 18)
Rooibos Fermented	14.5 ± 11.97 b[$] (329 ± 30)	82.61 ± 8.15 c* (163 ± 28)	94.12 ± 5.55 b** (132 ± 17)	98.12 ± 7.34 abcd[#] (122 ± 21)
Rooibos Unfermented		63.74 ± 13.48 g* (210 ± 34)	83.90 ± 13.99 f** (157 ±35)	93.59 ± 7.98 g[#] (133 ± 23)
Black tea	38.37 ± 10.83 a[#] (270 ± 27)	95.93 ± 6.64 a* (128 ± 23)	99.61 ± 5.71 a** (119 ± 21)	98.81 ± 5.36 a*,** (120 ± 17)
Oolong tea	31.70 ± 11.00 a[#] (287 ± 27)	88.65 ± 5.87 b* (147 ± 18)	97.23 ± 5.43 a** (124 ± 20)	98.79 ± 11.22 ab** (125 ± 17)
Green tea	33.4 ± 13.28 a[$] (283 ± 33)	81.82 ± 13.63 cd* (169 ± 46)	92.11 ± 8.20 bc** (140 ± 31)	97.72 ± 6.73 abcde[#] (123 ± 20)

[a] Concentration of aqueous extracts in mg per plate.

[b] The mean frequency of spontaneous reversion for the experiments with concentration level of 0.1 mg aqueous extract per plate was 118± 2 with the mean AFB_1-induced mutagenesis 365± 25.

[c] The mean frequency of spontaneous reversion for the experiments with 0.5, 1 and 2 mg extract aqueous extract per plate was 117± 13 with the mean AFB_1-induced mutagenesis 379± 42.

[d] Values represent the mean ± SD of percentage inhibition followed by the revertant count in brackets of six samples (n = 6) with five assay replications. Means in the same column followed by different letters are significantly different ($P < 0.05$).

[e] Means in the same row marked with different symbol (*, **, #, $) indicate a dose response effect with significant differences ($P < 0.05$) between the concentration levels for the same tea.

aqueous extract per plate, antimutagenic activity of *Camellia sinensis* teas differed significantly and decreased in the order black (95.93%) > oolong (88.65%) > green tea (81.82%). This trend was also observed at concentrations of 1 and 2 mg aqueous extract per plate and antimutagenic activity of green tea was significantly ($P < 0.05$) less than that of black and oolong teas at 1 mg per plate.

At a concentration level of 0.5 mg aqueous extract per plate the antimutagenic activity of fermented rooibos (82.61%) was similar ($P > 0.05$) to that of green tea (81.82%), while comparison at 0.1 mg per plate revealed that antimutagenic potency of fermented rooibos was significantly ($P < 0.05$) less than that of teas produced from *Camellia sinensis*. Fermented rooibos tea exhibited more potent antimutagenic activity at all the concentration levels than the unfermented samples, with a major difference evident at 0.5 mg aqueous extract per plate where fermented rooibos (82.61%) inhibited mutagenicity significantly more ($P < 0.05$) than unfermented rooibos (63.74%).

Fermentation significantly ($P < 0.05$) reduced antimutagenic activity of *Cyclopia spp.*, except *C. genistoides* at 0.5 and 1 mg aqueous extract per plate, where no significant difference was observed between the unfermented tea and its fermented counterpart. Fermented *C. intermedia* exhibited the least ($P < 0.05$) antimutagenic potency at 0.5 and 1 mg aqueous extract per plate when compared to the other *Cyclopia spp.* An increase in the concentration level of aqueous extracts resulted in a decline in the difference of antimutagenic activity of fermented and unfermented *Cyclopia spp.*, which was most prominent in *C. intermedia*. At 2 mg aqueous extract per plate antimutagenic activity of unfermented *C. intermedia*, *C. subternata* and *C. sessiliflora* was not significantly ($P < 0.05$) less than that of black, oolong and green or fermented rooibos teas.

Substantial variations in the antimutagenic activity were observed for samples of the same species and the percentage inhibition of AFB_1-induced mutagenesis at a concentration level of 0.5 mg aqueous extract per plate by the individual samples is presented in Table 8. The influence of natural plant variation on antimutagenicity was also demonstrated at concentration levels of 0.1, 1 and 2 mg aqueous extract per plate (Addendum B, Tables 1-3).

Significant differences in the antimutagenic activity of the individual samples were evident for many of the teas (Table 8). The most variation in plant material in terms of the influence on antimutagenic activity at a concentration level of 0.5 mg aqueous extract per plate was evident for unfermented rooibos tea (ranging from 52.16-85.34%) and fermented *C. subternata* (25.96-57.33%), *C. genistoides* (29.50-61.04%) and *C. sessiliflora* (28.62-61.04%) (Table 8).

Table 8 Effect of plant variation on antimutagenic activity of aqueous extracts of fermented and unfermented *Cyclopia spp.* and rooibos as well as *Camellia sinensis* teas at 0.5 mg aqueous extract per plate against AFB_1 (20 ng per plate) induced mutagenesis in *Salmonella typhimurium* TA100.

	Batches					
Tea	**1**	**2**	**3**	**4**	**5**	**6**
C. intermedia, f[a]	31.19 ± 12.56[b] abc	29.37 ± 17.59 ab	14.57 ± 7.09 de	17.31 ± 4.77 cde	17.60 ± 2.77 bcd	39.26 ± 1.94 a
C. intermedia,uf[a]	76.00 ± 4.11 bcd	76.71 ± 9.67 abc	74.66 ± 7.92 bcde	70.26 ± 1.45 ce	79.54 ± 5.71 ab	85.76 ± 2.70 a
C. subternata, f	33.47 ± 10.09 bcd	39.86 ± 10.21 bc	33.36 ± 8.44 bcde	25.96 ± 2.63 cde	47.66 ± 3.89 ab	57.33 ± 2.17 a
C. subternata,uf	64.63 ± 8.70 c	78.88 ± 5.28 ab	71.38 ± 9.89 bc	61.13 ± 7.71 c	86.25 ± 4.85 d	79.71 ± 2.23 ab
C. genistoides, f	58.19 ± 13.94 ab	42.36 ± 9.30 bcde	29.50 ± 10.21 ce	43.54 ± 3.39 bcd	61.04 ± 0.62 a	46.92 ± 12.82 abc
C. genistoides,uf	45.07 ± 6.87 bde	35.39 ± 6.47 de	49.48 ± 8.00 abc	48.25 ± 3.97 abcd	60.57 ± 5.06 a	55.85 ± 5.68 ab
C. sessiliflora, f	48.93 ± 9.03 a	28.94 ± 19.72 abcd	28.62 ± 10.73 cd	46.02 ± 5.90 ab	61.04 ± 4.78 e	41.14 ± 4.68 abc
C. sessiliflora, uf	78.18 ± 16.63 ab	79.15 ± 10.45 a	68.94 ± 13.37 abcd	63.19 ± 7.23 bde	64.91 ± 2.19 bde	75.81 ± 3.94 abc
Rooibos tea, f	88.21 ± 5.02 a	**79.32 ± 10.31** ab	**82.59 ± 7.00 ab**	**71.77 ± 3.37** b	**84.15± 3.86** a	89.65 ± 3.30 a
Rooibos tea, uf	61.04 ± 6.90 bc	58.40 ± 9.63 bcd	52.16 ± 13.20 cde	56.80 ± 6.40 cde	**73.04± 5.20** ab	**85.34 ± 7.51** a
Black tea, f	102.51 ± 5.20 a	102.51 ± 2.86 a	87.67 ± 5.21 c	91.28 ± 4.36 bc	95.60± 1.48 abc	96.04 ± 3.92 ab
Oolong tea, sf[a]	92.98 ± 8.39 a	86.99 ± 4.23 a	87.07 ± 2.54 a	89.35 ± 6.42 a	83.27 ± 2.81 a	92.27 ± 4.73 a
Green tea, uf	84.11 ± 10.36 abcde	95.97 ± 7.18 a	85.78 ± 5.18 abc	67.10 ± 6.15 cef	71.68 ± 18.92 abcdf	90.79 ± 5.90 ab

[a] f = fermented, uf = unfermented, sf = semi-fermented.

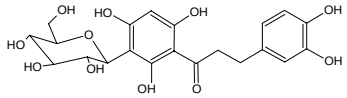
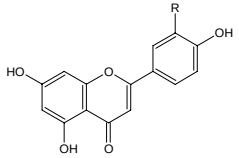
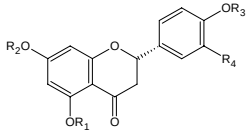
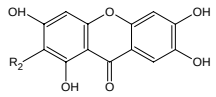
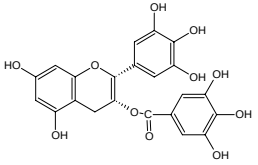
[b] Values represent the mean ± SD of percentage inhibition of AFB_1-induced mutagenesis determined with five assay repeats. Means in the same row followed by the different letters are significantly different ($P < 0.05$).

Antimutagenic acitivity of black tea samples exhibited less variation and inhibitions were between 87.67 and 102.51%, while unfermented *C. intermedia* (70.26-85.76%) and *C. sessiliflora* (63.19-79.15%) also demonstrated less variation in antimutagenic activity due to natural plant variation. Two of the unfermented rooibos tea samples demonstrated higher antimutagenic activity (indicated in bold, Table 8) than some of the fermented samples.

The protective effect of selected phenolic compounds at 0.1 and 0.3 mM per plate against AFB_1-induced mutagenesis in the *Salmonella* mutagenicity assay is summarised in Table 9. The antimutagenic activity of luteolin and chrysoeriol was similar to that of EGCG and hesperetin at a concentration level of 0.1 mM, however, at 0.3 mM, antimutagenic activity of EGCG and hesperetin was significantly ($P < 0.05$) higher than that of luteolin. The protective effect demonstrated by eriodictyol at 0.1 mM per plate was significantly ($P < 0.05$) less than luteolin and chrysoeriol, while at 0.3 mM it was similar to the antimutagenic activity of these two compounds and hesperetin. Hesperidin and mangiferin exhibited less antimutagenic activity at 0.3 mM than the other compounds, except aspalathin, which was the least effective. Antimutagenic activity of aspalathin was not determined at 0.1 mM since it demonstrated such low levels of inhibition at 0.3 mM per plate. Mangiferin had no antimutagenic activity against AFB_1 at 0.1 mM per plate, while hesperidin exhibited a very low level of inhibition (21.49%). An increased concentration resulted in significantly ($P < 0.05$) increased antimutagenic activity of all the compounds, except for luteolin and chrysoeriol (Table 9).

The individual concentration levels of selected phenolic compounds (nM per plate) in fermented and unfermented *Cyclopia spp.* (Table 10) and rooibos tea (Table 11) at a concentration of 0.5 mg aqueous extract per plate in the *Salmonella* mutagenicity assay, were calculated to gain insight regarding the levels of phenolic compounds in the antimutagenic assay. The levels of the respective compounds contained in the tea extracts were substantially lower than the concentration levels of 0.1 (100 000 nM) and 0.3 mM (300 000 nM) per plate used to determine antimutagenic activity of selected phenolic compounds individually. The concentration of mangiferin contained in the aqueous extract amounted to 4.256 nM mangiferin per plate for unfermented *C. genistoides,* containing the highest levels of mangiferin of the *Cyclopia spp.* at 10.04% of the aqueous extract. This was *ca*. 70 500 times and *ca*. 23 500 times less than the concentration levels of 300 000 nM and 100 000 nM per plate, respectively. The high levels of aspalathin (6.77% of aqueous extract) in unfermented rooibos tea only amounted to a concentration of 2.714 nM per plate (Table 11), which was *ca*. 110 000 times less than 300 000 nM per plate.

Table 9 Antimutagenic activity and structures of selected phenolic compounds of *Cyclopia spp.*, rooibos and *Camellia sinensis* teas against AFB_1 (20 ng per plate) in *Salmonella typhimurium* TA100 at concentration levels of 0.1 and 0.3 mM per plate.

Compound type	Structure	Compound	Substitution	Antimutagenicity 0.1 mM per plate[a]	Antimutagenicity 0.3 mM per plate[a]
Dihydro-chalcone		Aspalathin		not determined	30.43± 6.39 e (336±19)
Flavone		Luteolin	R=OH	85.64± 3.86 a (154± 10)	82.49± 1.01 c (185 ± 3)
		Chrysoeriol	R=OMe	84.91± 10.16 ab (156± 25)	85.64± 3.86 bc (175 ± 15)
Flavanone		Eriodictyol	R_1=R_2=R_3=H, R_4=OH	60.50± 10.2*[b] cd (216± 25)	88.39± 3.98* abc (168 ± 12)
		Hesperidin	R_1=H, R_2=rutinosyl, R_3=Me, R_4=OH	21.49± 7.05* e (333± 27)	69.44± 3.66* d (223± 11)
		Hesperetin	R_1=R_2=H, R_3=Me, R_4=OH	73.64± 9.89* abcd (175± 16)	91.55± 4.6* ab (158± 13)
Xanthone		Mangiferin	R_2=2-β-*D*-glucopyranosyl	-1.01± 13* f (368± 32)	66.35± 5.36* d (232±16)
Flavanol (Catechin)		EGCG		78.10± 7.69* abc (172± 19)	95.40± 2.70* a (147± 8)

[a] Values represent the means ± SD of percentage inhibition followed by the revertant count in brackets of five assay replications. Means in the same column followed by different letters are significantly ($P < 0.05$) different.

[b] Means within the same row marked with a symbol (*) indicate a significant ($P < 0.05$) dose response effect.

[c] The frequency of spontaneous reversion was (a) 118 ± 2 (negative control) and 365 ± 27 (positive control) for the *Salmonella* mutagenicity assay with 0.1 mM per plate and 134 ± 6 (negative control) and the 425 ± 11 (positive control) with 0.3 mM per plate.

Table 10 Concentration (nM per plate) of mangiferin and hesperidin in aqueous extracts prepared from fermented and unfermented *Cyclopia spp.* at a concentration level of 0.5 mg aqueous extract per plate in the *Salmonella* mutagenicity assay.

	Mangiferin[a]	**Hesperidin**[a]
C. intermedia Fermented	0.097	0.067
C. intermedia Unfermented	1.057	0.329
C. subternata Fermented	0.043	0.092
C. subternata Unfermented	0.503	0.122
C. genistoides Fermented	1.818	0.137
C. genistoides Unfermented	4.256	0.270
C. sessiliflora Fermented	0.076	0.131
C. sessiliflora Unfermented	1.862	0.145

[a] Values represent the mean concentration (nM per plate) of six samples (n = 6) calculated using the quantitative (as percentage of aqueous extract) values determined with reversed-phase HPLC analysis carried out in duplicate on a Synergy MAX-RP C12 column with acetonitrile and 2% acetic acid in water as eluents.

Table 11 Concentration (nM per plate) of aspalathin, orientin and iso-orientin in aqueous extracts prepared from fermented and unfermented rooibos tea at a concentration level of 0.5 mg per plate in the *Salmonella* mutagenicity assay.

	Aspalathin[a]	**Orientin**[a]	**Iso-orientin**[a]
Rooibos Fermented	0.261	0.280	0.343
Rooibos Unfermented	2.714	0.232	0.258

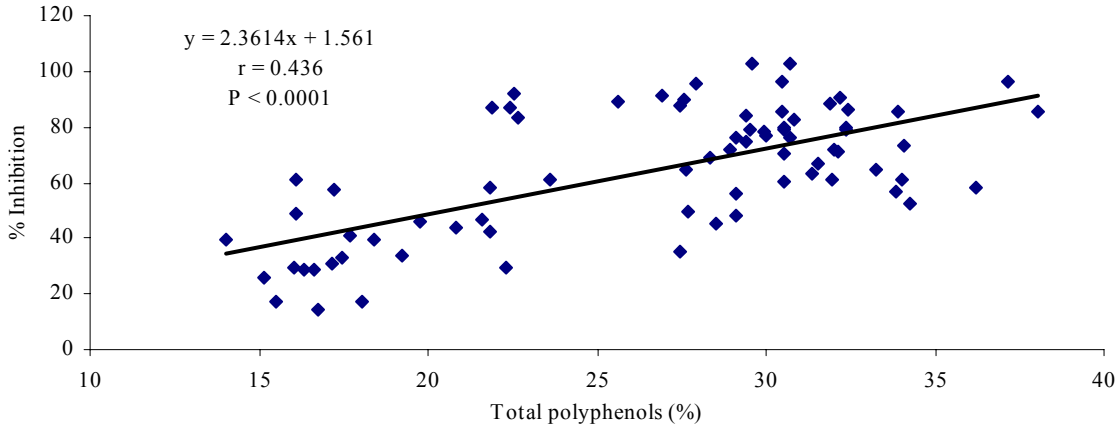
[a] Values represent the mean concentration (nM per plate) of six samples calculated from the quantitative (as a percentage of aqueous extract) values determined with HPLC on a reversed phase LiChrospher 100 RP–18 C_{18} column with 2% formic acid in water and 100% methanol as eluents.

Correlation of antimutagenic activity with phenolic composition of aqueous extracts

The overall correlation of the percentage inhibition of AFB_1-induced mutagenesis (antimutagenic activity) with the total polyphenol, flavanol and flavonol/flavone contents of the aqueous extracts respectively is depicted in Fig. 3. Antimutagenic activity of the teas correlated moderately ($r = 0.551$, $P < 0.0001$) with the flavanol content and to a lesser extent with the total polyphenol content ($r = 0.436$, $P < 0.0001$). The flavonol/flavone content of the aqueous extracts did not correlate ($r = 0.004$, $P = 0.58$) with antimutagenic activity.

Correlation of antimutagenic activity of the respective teas, i.e. *Cyclopia spp.*, rooibos and *Camellia sinensis,* with the level of the different phenolic groups, is depicted in Fig. 4 and summarised in Table 12 (correlations indicated in bold). Antimutagenic activity of *Cyclopia spp.* correlated strongly ($r = 0.805$, $P < 0.0001$) with its total polyphenol content (Fig 4a) and moderately ($r = 0.653$, $P < 0.0001$) with its flavanol content (Fig. 4b), but did not correlate with the flavonol/flavone content (Table 12). The total polyphenol content of *Cyclopia spp.* could clearly be distinguished (higher cluster) from the unfermented teas as is illustrated in Fig. 4a. A moderate correlation ($r = 0.751$, $P = 0.005$) was evident between the flavonol/flavone content of aqueous extracts from rooibos tea and its antimutagenic activity, with two separate clusters observed for fermented and unfermented samples (Fig 4c). No correlation of antimutagenicity with the total polyphenol or flavanol content existed (Table 12). The antimutagenic activity of *Camellia sinensis* teas did not correlate with the phenolic content of the aqueous extracts (Table 12). The correlation of the levels of the major individual compounds in *Cyclopia spp.* and rooibos tea, with the antimutagenic activity of their extracts is presented in Fig. 5 and summarised in Table 13 (correlations indicated in bold). A strong negative correlation ($r = -0.918$, $P < 0.0001$) existed for the aspalathin content of rooibos tea with its antimutagenic activity (Fig. 5a). Correlation of the iso-orientin content with the antimutagenicity of rooibos yielded a correlation coefficient of 0.728 ($P < 0.011$), which was higher than that for orientin ($r = 0.674$, $P = 0.023$) (Figs. 5b and c). An outlier, evident in Fig. 5, was not included in the calculation of the correlation of aspalathin, orientin and iso-orientin with antimutagenicity of rooibos tea. No correlation existed for antimutagenic activity of *Cyclopia spp.* with its mangiferin content, while antimutagencity showed a weak ($r = 0.363$), but significant ($P = 0.012$) correlation with the hesperidin content.

a)



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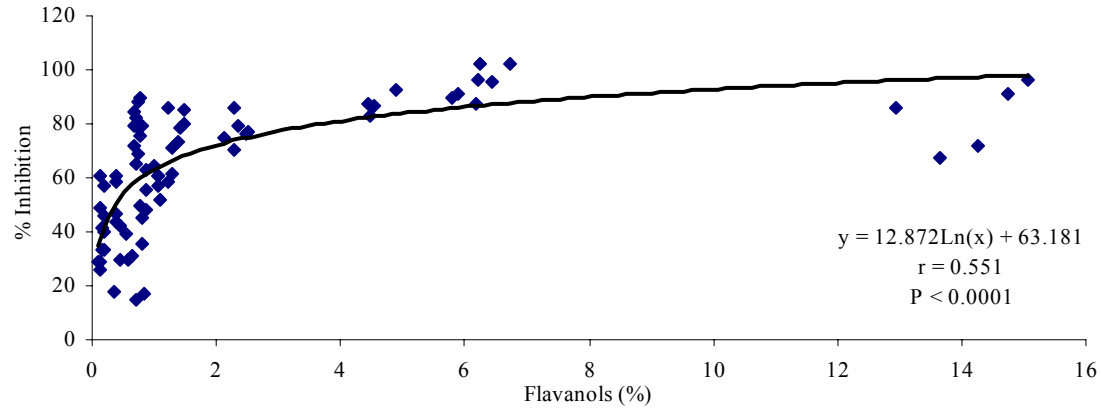

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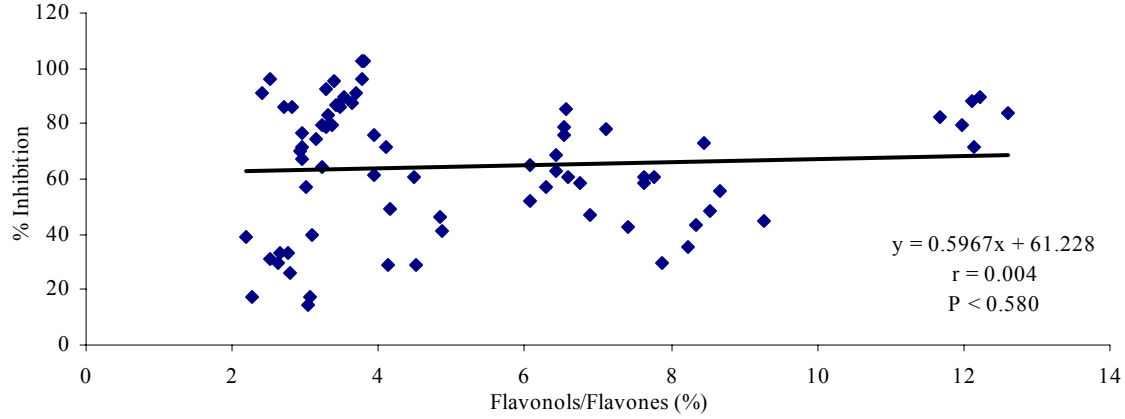
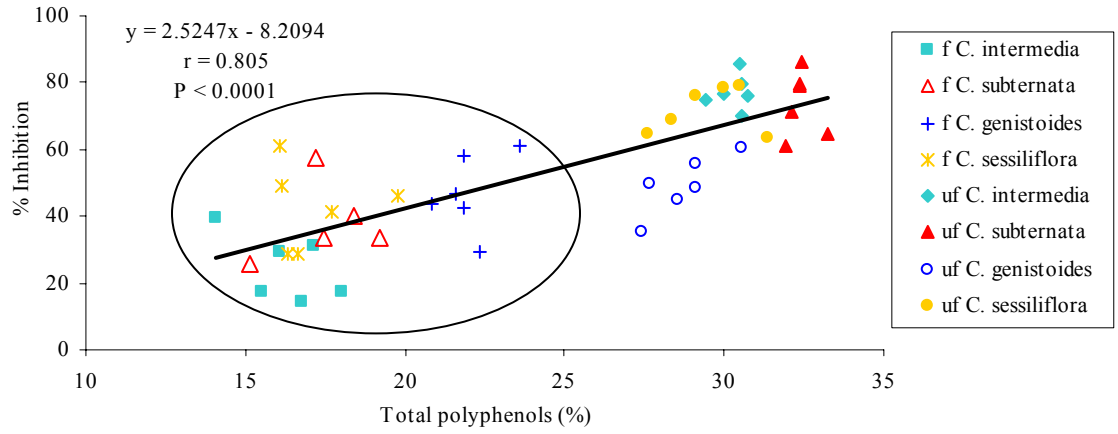
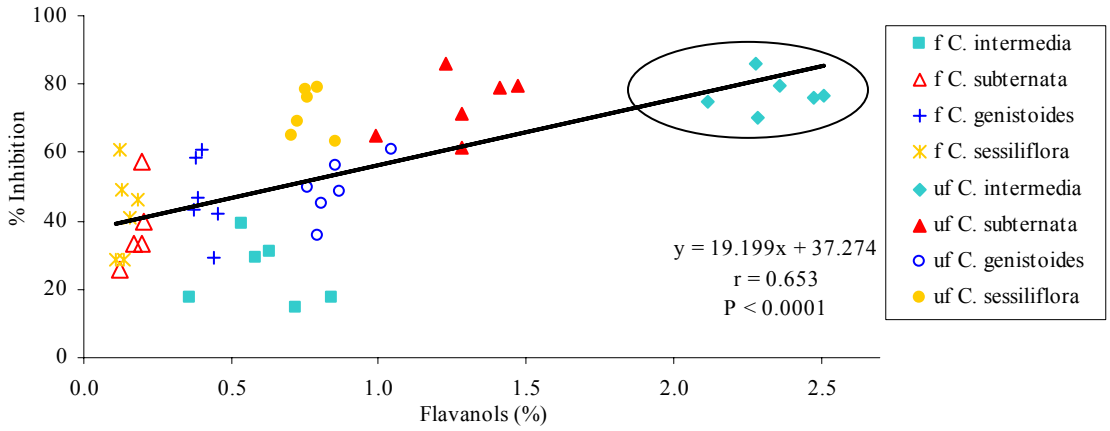


Figure 3 Overall correlation of antimutagenic activity (% inhibition) of fermented and unfermented *C. intermedia*, *C. subternata, C. genistoides* and *C. sessiliflora* and fermented and unfermented rooibos at a concentration of 0.5 mg aqueous extract per plate and *Camelia sinensis* teas at 0.1 mg per plate against AFB_1 in the *Salmonella* mutagenicity assay with (a) total polyphenol, (b) flavanol and (c) flavonol/flavone contents of their aqueous extracts.

a)



b)



c)

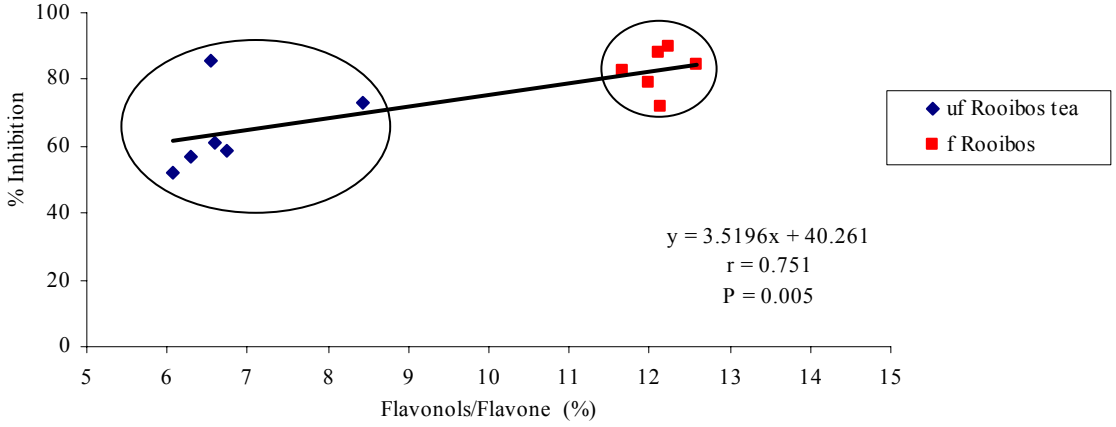


Figure 4 Correlation of antimutagenic activity (% inhibition) of fermented (f) and unfermented (uf) *C. intermedia*, *C. subternata*, *C. genistoides* and *C. sessiliflora* with its a) total polyphenol and b) flavanol contents and the correlation of antimutagenicity (% inhibition) of fermented and unfermented rooibos with its c) flavonol/flavone content.

Table 12 Separate correlation of antimutagenic activity[a] of fermented and unfermented *Cyclopia spp.*, fermented and unfermented rooibos and *Camellia sinensis* teas with the levels of phenolic groups in the aqueous extracts.

Tea	Total Polyphenols (g gallic acid equivalents 100 g^{-1})	Flavanols (g catechin equivalents 100 g^{-1})	Flavonol/Flavones (g quercetin equivalents 100 g^{-1})
Cyclopia spp.[b]	**0.805[c] (P < 0.0001)**	**0.653 (P < 0.0001)**	0.063 (P = 0.675)
Rooibos tea[d]	-0.466 (P = 0.127)	-0.397 (P = 0.201)	**0.751 (P = 0.005)**
Camellia sinensis[e]	0.276 (P = 0.301)	0.003 (P = 0.993)	0.306 (P = 0.249)

[a] Antimutagenic activity expressed as percentage inhibition at a concentration level of 0.5 mg aqueous extract for *Cyclopia spp.* and rooibos and 0.1 mg aqueous extract per plate for *Camellia sinensis* teas against metabolically activated AFB_1 (20 ng per plate).

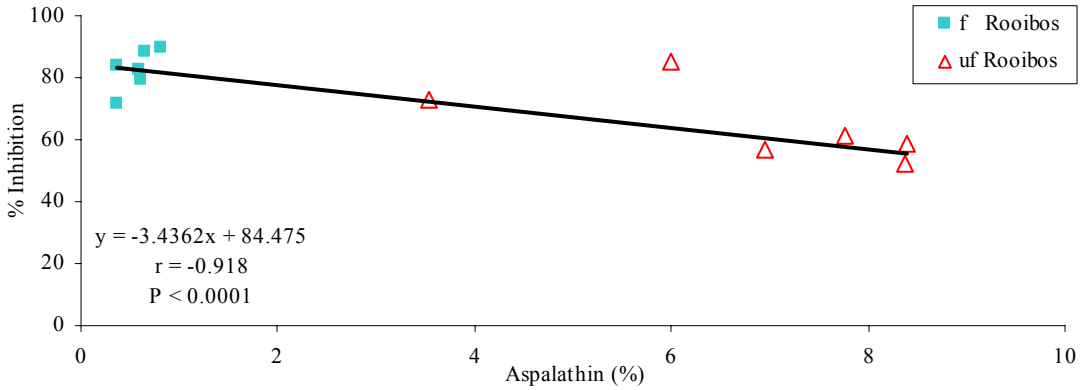
[b] The correlation of levels of phenolic groups in *Cyclopia spp.* with antimutagenic activity was determined for fermented and unfermented *C. intermedia, C. subternata, C. genistoides* and *C. sessiliflora.*

[c] Pearson correlation coefficient of the correlation of total polyphenol, flavanol and flavonol/flavone with antimutagenic activity of the different tea groups, followed by the probability in brackets.

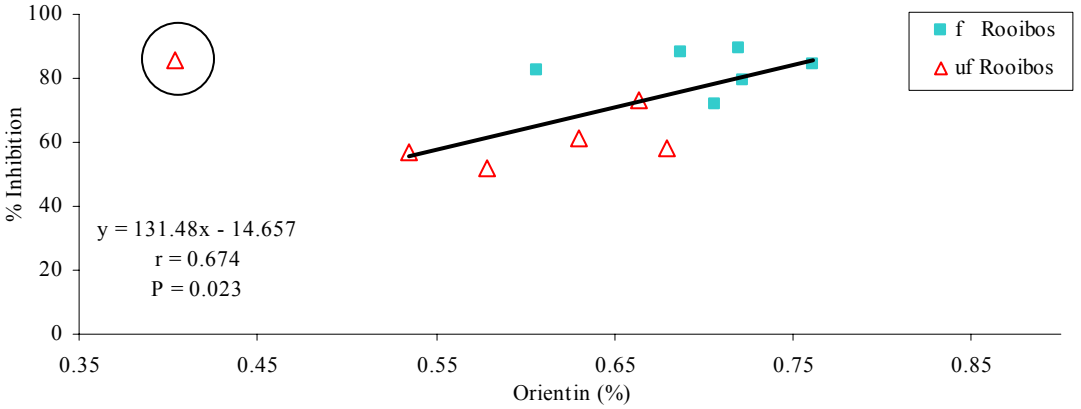
[d] The correlation of levels of phenolic groups in rooibos tea with antimutagenic activity was determined for fermented and unfermented *Aspalathus linearis*.

[e] The correlation of levels of phenolic groups in *Camellia sinensis* teas with antimutagenic activity was determined for black, oolong and green teas.

a)



b)



c)

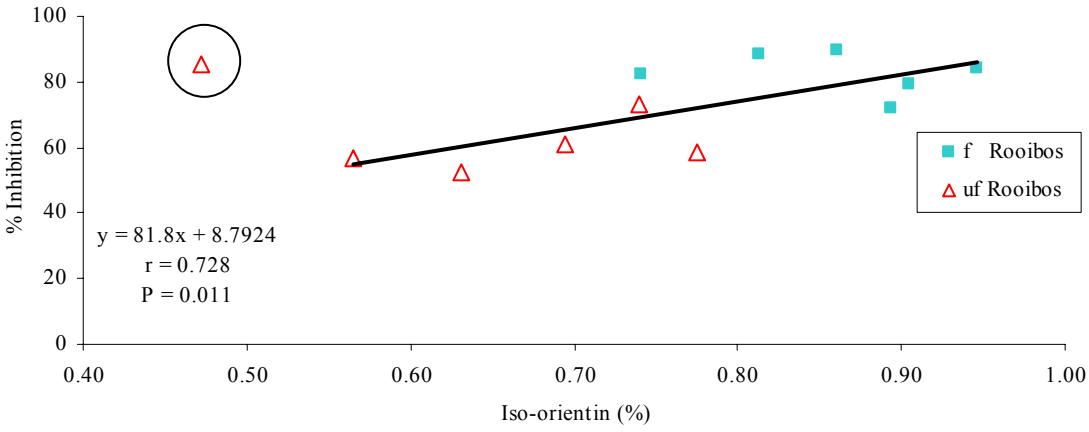


Figure 5 Correlation of antimutagenicity of fermented (f) and unfermented (uf) rooibos tea against AFB_1-induced mutagenesis with a) aspalathin b) orientin and c) iso-orientin content of the individual samples. An outlier, circled on the graphs, was not included in correlation analysis of orientin and iso-orientin.

Table 13 Separate correlation of antimutagenic activity[a] of aqueous extracts of fermented and unfermented *Cyclopia spp.* and fermented and unfermented rooibos with the levels of the major compounds in the respective aqueous extracts.

Tea	Aspalathin[b]	Orientin[b]	Iso-orientin[b]	Mangiferin[c]	Hesperidin[c]
Cyclopia spp[d].				0.153[e] (P = 0.304)	**0.363 (P = 0.012)**
Rooibos tea[f]	**-0.918 (P < 0.0001)**	**0.674 (P = 0.023)**	**0.728 (P = 0.011)**		

[a] Antimutagenic activity expressed as percentage inhibition at a concentration level of 0.5 mg for *Cyclopia spp.* and rooibos against metabolically activated AFB_1 (20 ng per plate).

[b] Aspalathin, orientin and iso-orientin content of fermented and unfermented rooibos tea determined with HPLC on a reversed phase LiChrospher 100 RP–18 C_{18} column with 2% formic acid in water and 100% methanol as eluents, expressed as a percentage of the aqueous extracts. An outlier was removed from the data for unfermented rooibos for calculation of correlations with orientin and iso-orientin content.

[c] Mangiferin and hesperidin content of fermented and unfermented *Cyclopia spp.* determined with reversed-phase HPLC analysis carried out on a Synergy MAX-RP C12 column with 100% acetonitrile and 2% acetic acid in water as eluents, expressed as a percentage of the aqueous extracts.

[d] The correlation of levels of phenolic compounds in *Cyclopia spp.* with antimutagenic activity was determined for fermented and unfermented *C. intermedia, C. subternata, C. genistoides* and *C. sessiliflora.*

[e] Pearson correlation coefficient of the correlation of compounds with antimutagenic activity of the aqueous extracts followed by the probability in brackets.

[f] The correlation of levels of phenolic compounds in rooibos tea with antimutagenic activity was determined for fermented and unfermented *Aspalathus linearis.*

Discussion

Antimutagenic activity of *Camellia sinensis* teas has mostly been investigated in microbial and mammalian *in vitro* and *in vivo* systems (Kuroda & Hara, 1999). The *Salmonella* mutagenicity assay was specifically designed for detection of chemically induced mutagenesis (Ames *et al*., 1975), but has been utilised extensively in valuation of antimutagenic activity of *Camellia sinensis* teas (Wang *et al*., 1989; Yen & Chen, 1996; Bu-Abbas *et al*., 1997; Chen & Yen, 1997; Yang *et al*., 2001) and recently of two herbal teas indigenous to South Africa, i.e. *Cyclopia spp.* (honeybush) (Marnewick *et al*., 2000; Richards, 2002) and *Aspalathus linearis* (rooibos) (Standley, 1999; Marnewick *et al*., 2000). Marnewick *et al*. (2004a) has demonstrated that liver cytosolic fractions from rats that had received fermented and unfermented rooibos and *C. intermedia*, protected against AFB_1-induced mutagenesis *ex vivo*, while green and black tea showed no cytocolic protection.

This investigation presents the first comparative study on *in vitro* antimutagenic activity of commercially important *Cyclopia spp.*, rooibos and *Camellia sinensis* teas, conducted to gain perspective on antimutagenic potency of rooibos and honeybush tea. The present study also provides the first data on the antimutagenic activity of *C. subternata, C. genistoides* and *C. sessiliflora* against AFB_1-induced mutagenesis in the *Salmonella* mutagenicity assay with tester strain TA100.

In the present study fermentation resulted in increased antimutagenic activity of *Camellia sinensis* teas in the order green (unfermented) < oolong (semi-fermented) < black (fermented). The overall results on antimutagenic activity of green and black tea in the literature, however, indicate that antimutagenic activity does not differ significantly, but reports on black tea are few in comparison (Gupta *et al*., 2002a). Yen & Chen (1994) reported that the antimutagenic activity of oolong was higher than that of black and green tea against 2-amino-3-methylimidazo(4,5-f)quinolin, 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole, 2-amino-6-methyldipyrido(1,2-a:3',2'-d)imidazole, benzo[a]pyrene (B[a]P) and AFB_1 using tester strain TA98 and TA100 in the presence of S9. The higher antimutagenic activity of semi-fermented tea was attributed to the formation of antimutagenic substances during manufacturing processes of tea (Yen & Chen, 1994).

While overall antimutagenic activity of the teas (*Camellia sinensis*, rooibos and *Cyclopia spp.*) correlated ($r = 0.436$, $P < 0.0001$) with the total polyphenol content of the aqueous extracts, correlation of the separate teas revealed that antimutagenic activity of *Camellia sinensis* teas

against metabolically activated AFB_1 did not correlate with the flavanol (catechins), total polyphenol or flavonol/flavone contents. Yen & Chen, 1996 demonstrated that antimutagenic activity of green tea against benzo[a]pyrene (B[a]P) and AFB_1, using *Salmonella* tester strain **TA98,** correlated significantly with the EGCG and total polyphenol contents. Antimutagenicity of green tea against 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) mutagencity in *Salmonella* tester strain TA100 correlated strongly ($r = 0.976$, $P < 0.0.12$) with the total catechin and EGCG contents ($r = 0.997$, $P < 0.0001$) (Yen & Chen, 1996). In contrast, no significant correlations were evident for the total flavanol or individual flavanol contents, or total polyphenol content of extracts prepared from green tea with antimutagenic activity against B[a]P or AFB_1 with tester strain **TA100** (Yen & Chen, 1996). This indicates that correlations and comparisons for antimutagenic activity of tea with the phenolic composition should be interpreted with care, and that activity varies with the mutagen type and tester strain used.

The high flavanol content of green tea, comprising approximately 30% of the total dry weight (Balentine *et al.*, 1997), has led to it being considered the most likely component responsible for its anticarcinogenic and antimutagenic properties (Bu-Abbas *et al*., 1997; Wang *et al*., 2000). Flavanols in green tea may contribute towards the ability of green tea to trap the reactive intermediates of certain chemical carcinogens (Bu-Abbas *et al*., 1997). EGCG, the most abundant flavanol in green tea, is known to posess potent antimatugenic activity against several mutagens, including, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and B[a]Pdiol oxide in tester strains TA98 and TA100, respectively (Kuroda & Hara, 1999). Thearubigins, assumed to be heterogenous polymers, are the most abundant polyphenolic oxidation products in black tea and it is estimated that up to 75% of the flavanols (catechins) present in green tea may be converted to thearubigins during the manufacturing of black tea (Haslam, 2003). This group of phenolic compounds constitute up to 20% of the black tea leaf, while the other important group of phenolic compounds in black teas, theaflavins, constitute only *ca.* 2% of the dry weight of black tea (Haslam, 2003). The unchanged flavanol content is between 1 and 3% of the dry weight (Lunder, 1992).

Antimutagenic activity of theaflavins and thearubigins have been demonstrated at different concentrations against sodium azide, 4-nito-o-phenylenediamins, cumine hydroperoxide, 2-aminofluorene and danthron in *Salmonella* tester strains TA97a, TA98, TA100 and TA102 (Gupta *et al.,* 2002b). Although black tea theafulvins possess potent antimutagenic activity against food carcinogens such as polycyclic aromatic hydrocarbons, nitrosamines and heterocyclic amines (Catterall *et al*., 1998), it has been demonstrated to stimulate the

mutagenicity of AFB_1 in a dose-dependent manner (Catterall *et al*., 2003). Experimental evidence thus indicate that in addition to monomeric flavanols, e.g. EGCG, which are more abundant in green tea than in black tea (Graham, 1992), other oligomeric flavanols present in fermented teas may be responsible for strong biological effects (Shi *et al*., 1994). Similar antimutagenic activity of green and black teas, despite the markedly different flavanol content, indicates that flavanols are unlikely to be the principle component responsible for antimutagenic activity of green tea, or that black tea polyphenols such as theaflavins and thearubigins are similarly active in this respect (Bu-Abbas *et al.*, 1996).

The *Camellia sinensis* teas were more effective antimutagens than rooibos and *Cyclopia spp.* This is attributed to vast differences in composition illustrated in the present study, e.g. flavanols are the major monomeric compounds in *Camellia sinensis* teas, whereas the major monomeric polyphenols in rooibos comprise a dihydrochalcone, aspalathin and the flavones, orientin and iso-orientin. The major monomeric compounds in *Cyclopia spp.* encompass the xanthone, mangiferin and the flavanone, hesperidin. The contribution of the major phenolic compounds to antimutagenic activity will be discussed.

An additional major difference in the composition of the teas is the presence of high levels of caffeine in *Camellia sinensis*, which is absent in rooibos (Blommaert & Steenkamp, 1978) and honeybush teas (Greenish, 1881). This compound has, however, been excluded as antimutagen at the concentrations encountered in tea, ranging between 22 to 30 mg 100 mL^{-1} in different green teas (Wang *et al*., 2000), based on the observation that black tea and decaffeinated black tea display similar antimutagenicity (Bu-Abbas *et al*., 1996).

Fermented rooibos tea exhibited higher protection against AFB_1 than unfermented rooibos tea in the present study. This was similar to the findings of Richards (unpublished data, 2002) using 2-AAF as mutagen. Another study by Standley *et al.* (2001), however, clearly demonstrated that fermentation significantly ($P < 0.05$) reduced antimutagenic potency of rooibos tea against 2-AAF using tester strain TA98 for 10 random samples collected before and after fermentation. It was clearly demonstrated in the present study that significant ($P < 0.05$) differences exist in antimutagenic activity of different samples of plant material within the same species. Although the average antimutagenic activity of fermented rooibos tea was higher than that of the unfermented tea, two unfermented samples displayed higher antimutagenic activity than some of the fermented samples. Further investigation including more samples, is recommended to establish whether fermented rooibos is indeed a more potent antimutagen

against AFB_1 than unfermented, and if the results obtained here or in other studies are an effect of sample selection.

Antimutagenic activity of rooibos tea did not correlate with its total polyphenol or flavanol content, while a relatively strong correlation ($r = 0.751$, $P < 0.005$) was evident for the flavonol/flavone content. This effect is attributed to the flavonol/flavone content of rooibos tea roughly doubling (from 6.82% to 12.12% of the aqueous extract) with fermentation, indicating that it may be the important phenolic group responsible or at least partly, for antimutagenic activity of fermented rooibos. Standley *et al*. (2001) attributed the decrease in antimutagenic activity of rooibos against 2-AAF to the reduction in the tea polyphenol content during fermentation. In contrast, the significant ($P < 0.05$) decrease in the total polyphenol content of rooibos tea with fermentation, in the present study, was not associated with antimutagenic activity against AFB_1.

The significant correlations of orientin ($r = 0.674$, $P < 0.0231$) and iso-orientin ($r = 0.728$, $P < 0.011$) content of aqueous extracts from rooibos tea with antimutagenic activity displayed against AFB_1, implies that these compounds might play a role in antimutagenic activity of rooibos tea. Antimutagenic activity of orientin and iso-orientin has, however, been reported to be similar to that of aspalathin compared at different concentrations against AFB_1 in the *Salmonella* mutagenicity assay with tester strain TA100 (Snijman *et al*., 2004).

The aspalathin content of 26 aqueous extracts from unfermented rooibos tea determined as part of a study to investigate seasonal variation, has been reported to be between 8.30 and 18.80% of the soluble solids (M. Botha, E. Joubert, M. Manley, unpublished data). This is much higher than the average aspalathin content of 6.77% reported in the present study. The contents of the individual samples varied between 3.49 and 8.29%, illustrating the variation, in terms of composition, in plant material. The nothofagin content of the rooibos samples tested in the present study was too low to quantify, but the average nothofagin content of unfermented rooibos has been reported to vary between 0.72 and 3.80% (M. Botha, E. Joubert, M. Manley, unpublished data). Oxidation of polyphenols is initiated with communution of the tea during processing and the decrease in dihydrochalcones (aspalathin and nothofagin) is attributed to enzymatic and chemical oxidation of polyphenols (Joubert, 1996). Joubert (1996) reported that the conversion of the dihydrochalcone, aspalathin to the intermediate reaction products, 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin and precursors of iso-orientin and orientin, observed in an ethanolic solution (Koeppen & Roux, 1966, Marais *et al*., 1998) probably occurs

in rooibos tea during fermentation, which could possibly explain the increase in iso-orientin and orientin contents with fermentation.

Fermentation resulted in significantly ($P < 0.05$) reduced antimutagenic activity for *Cyclopia spp.* at the three concentration levels tested against metabolically activated AFB_1, except for *C. genistoides*, which displayed similar antimutagenic activity before and after fermentation at 0.5 and 1 mg aqueous extract per plate. This is in agreement with the result of Richards (2002), demonstrating that fermentation decreases antimutagenic activity of *Cyclopia spp.* against 2-AAF, except for *C. genistoides*. Although unfermented *C. genistoides* exhibited significantly less antimutagenic potency than the other unfermented species tested, it did not enhance the mutagenicity as found with metabolically activated 2-AAF as mutagen (Richards, 2002). Interestingly, increasing the concentration of aqueous extract in the *Salmonella* mutagenicitiy assay resulted in significantly ($P < 0.05$) higher antimutagenic activity of fermented *C. genistoides* than its unfermented counterpart at 2 mg per plate.

Whilst the total polyphenol content of *Cyclopia spp.* correlated strongly ($r = 0.805$, $P < 0.0001$) with antimutagenic activity, implicating the importance of the levels of this phenolic group against metabolically activated AFB1 , no correlation (-0.082, $P = 0.563$) was evident with antimutagenic activity against 2-AAF (Richards, 2002). Similarly, the moderate correlation ($r = 0.653$, $P < 0.0001$) of protection against AFB_1 with the flavanol content, implicates a contribution to the protection against AFB_1, while Richards (2002) reported no ($r = 0.120$, $P = 0.395$) contribution of flavanols to protection of aqueous extracts against 2-AAF. Although the flavanol content of unfermented *C. intermedia* was significantly higher than that of other fermented and unfermented *Cyclopia spp.,* antimutagenic activity of unfermented *C. intermedia* was similar ($P > 0.05$) to that of unfermented *C. subternata* and *C. sessiliflora.* This may be interpreted that the levels of flavanols are not solely responsible for antimutagenic activity against AFB_1, and levels of other compounds and synergistic effects of phenolic compounds may be of importance. Flavonol/flavones did not appear to be of importance in antimutagenic activity of *Cyclopia spp.* against AFB_1 ($r = 0.063$, $P = 0.675$), while the flavonol/flavones exhibited a significant correlation ($r = -0.600$, $P < 0.0001$) with antimutagenic activity against 2-AAF (Richards, 2002). This indicates that different phenolic groups may be involved in antimutagenic activity of *Cyclopia spp.* against different mutagens, and may be attributed to differences in the active metabolites of AFB_1 and 2-AAF formed upon metabolic activation by the S9 mixture. Differences in polarity and structure of these metabolites would result in diverse interactions with phenolic groups. Another consideration would be that different phenolic groups are of

importance in the interference with metabolic activation, due to differential mechanisms of mutagen activation by cytochrome P450.

The aqueous extracts prepared from *Cyclopia spp.* previously analysed by Richards (2002) were re-analysed, with a different solvent system and using a C_{12} instead of the reversed-phase C_{18} column (Richards, 2002), in an attempt to separate mangiferin and isomangiferin, as well as to quantify additional compounds. Isomangiferin eluted separately, with the solvent system used, but could not be quantified due to the low levels present. Additional compounds quantified in *Cyclopia spp.* for the first time included eriocitrin in *C. subternata* and *C. sessiliflora* and unfermented *C. genistoides*, and narirutin in all the species, except *C. genistoides*. The level of 10.04% mangiferin reported in unfermented *C. genistoides* (West Coast type) in the present study is in agreement with the 9.69% that has been determined for 88 unfermented samples of West Coast type *C. genistoides* (M. Botha, E. Joubert, M. Manley, unpublished data), while the hesperidin levels for the same samples were much higher at 2.83% (M. Botha, E. Joubert, M. Manley, unpublished data) than that of 0.92% for the samples in the present study. The latter study was undertaken to investigate the seasonal variation in hesperidin content in West Coast type *C. genistoides.*

The levels of hesperetin were found to be significantly lower than that of mangiferin, hesperidin and eriocitrin (where present) in this study, with the highest levels present in fermented *C. intermedia.* De Nysschen *et al.* (1996) estimated that hesperetin is one of the major flavonoids present in unprocessed *Cyclopia spp.* and the presence of a large quantity of hesperetin has been reported in methanolic extracts of fermented *C. intermedia* (Ferreira *et al*., 1998). Hesperetin could not be detected in *C. subternata*, which is in agreement with the findings of Kamara *et al*. (2004), who reported the flavanones present in unfermented *C. subternata* as hesperidin, narirutin and eriocitrin.

Antimutagenic activity of *Cyclopia spp.* did not correlate significantly with mangiferin content, while a very weak, but significant correlation ($r = 0.363$, $P < 0.012$) of antimutagenic potency with the hesperidin content was observed. This was in agreement with the findings of Richards (2002), who reported that the levels of mangiferin/isomangiferin (quantified together using mangiferin as standard) did not appear to influence the antimutagenic activity of *Cyclopia spp.* against 2-AAF, but that a very weak ($r = 0.29$), but significant ($P = 0.033$) correlation existed for antimutagenic activity with the hesperidin content of *Cyclopia spp.*

The very high mangiferin and relatively high hesperidin content of unfermented *C. genistoides* did not seem to play a role in antimutagenic activity as this species exhibited weaker

antimutagenic activity than all the other unfermented species. The present study indicated that the number of samples included in investigation of biological properties may influence results in terms of activity, since such vast variations have been observed in terms of composition of plant material within the same species. The different phenolic profiles of the respective *Cyclopia spp.* may play a role in the relative antimutagenic potency against AFB_1. Relatively large quantities of unidentified compounds present in *C. intermedia*, *C. subternata* and *C. sessiliflora* could also play a role in antimutagenic activity. Richards (2002) also reported the presence of unidentified compounds in *C. intermedia* and *C. subternata*, which were substantially reduced with fermentation. The lack of correlation of antimutagenic activity with the levels of major compounds of *Cyclopia spp* and the very weak correlation for hesperidin, indicate that phenolic compounds with high molecular weight, not quantified with HPLC analysis, and other non-flavonoids present in the tea, may be the major compounds responsible for the antimutagenic activity present in *Cyclopia spp*. Marnewick *et al*. (2004b) reported that differences in the flavanol/proanthocyanidin (ButOH-HCl reactive substances) and flavonol/flavones composition, as well as non-polyphenolic constituents, are likely to be of importance in the inhibition of tumour promotion by ethanol/acetone soluble fractions of rooibos and *C. intermedia.*

Antimutagenic activity of selected phenolic compounds compared at the same concentration (molarity base) against metabolically activated AFB_1 in the *Salmonella* mutagenicity assay, demonstrated that both aspalathin and mangiferin are less active than the other compounds tested. Snijman *et al*. (2004) reported *ca.* 30% inhibition of 2-AAF-induced mutagenesis by aspalathin with tester strain TA98 at the different concentrations in the *Salmonella* mutagenicity assay. Hesperidin exhibited a much weaker antimutagenic activity against AFB_1 than its corresponding aglycone, hesperetin. Edenharder *et al*. (1993) and Edenharder & Tang (1997) reported that most flavonoid glycosides are not active antimutagens against heterocyclic amines and nitroarenes and that reduced antimutagenic acitivity is possibly explained by attachment of large polar substituents (glycosides) to flavonoids. Distinct structure-antimutagenicity relationships against a number of mutagens have been demonstrated for several flavonoids (Francis *et al*., 1989; Edenharder *et al*., 1993; Das *et al*., 1994; Edenharder & Tang, 1997; Edenharder *et al*., 1997). The physiological activities of flavonoids are strongly affected by their glycosylation and hydroxylation pattern, as well as the type of C2-C3 bond (Das *et al*., 1994). A slight change in the chemical structure may result in varying degrees of reactivity and a change in the pattern of glycosylation, hydroxylation and methylation, or the presence or absence of the C2-C3 double bond may lead to variations in solubility, absorption, resonance contributions, ionization

constants, metal chelation and thus biological activity (Das *et al*., 1994). *In vitro* antimutagenic activity of flavones against AFB_1 investigated in the present study did not appear to be influenced by the presence of hydroxyl groups, and luteolin (4 OH groups) exhibited similar antimutagenic activity to chrysoeriol (3 OH groups and a methoxy group). Flavonoid glycosylation appeared to be the main determinant in antimutagenic acitivity against AFB_1 of the compounds investigated here, as the glycosides aspalathin, hesperidin and mangiferin exhibited relatively weak antimutagenic activity.

Antimutagenic activity of structurally diverse flavonoids, the flavanol, EGCG, the flavanones, hesperetin and eriodictyol and the flavones, luteolin and chrysoeriol, did not differ significantly ($P > 0.05$) against AFB_1 at the concentration levels tested in this study. This can possibly be explained by the high levels of inhibition, mostly higher than 70% at 0.1 mM and higher than 80% at 0.3 mM per plate, by these compounds, resulting in the inability to distinguish between antimutagenic potency. Investigation of lower concentration levels in the *Salmonella* mutagenicity assay is recommended, to assist in determination of differences in antimutagenic potency of the major compounds in the respective teas investigated here.

An important consideration when comparing the antimutagenic activity of compounds at the same concentration level (molarity base), is that the relative activities cannot be taken as an indication of the contribution of these compounds to antimutagenic activity demonstrated by the aqueous extracts from the different teas. Comparison at the same concentration level does not take the actual levels of compounds in the tea extract as incorporated into the assay into account, nor are synergistic mechanisms considered. It was illustrated in the present study that the levels of mangiferin, as part of the tea extract from unfermented *C. genistoides*, was *ca*. 70 500 times less per plate than the concentation level of 0.3 mM used to compare the antimutagenic activity of selected phenolic compounds.

Conclusions

This study indicates that rooibos and *Cyclopia spp.* have *in vitro* antimutagenic activity against AFB_1, although less than black tea, suggesting that consumption of these two herbal teas may have beneficial health effects. Compositional differences between the teas, and the effect of fermentation on composition, highlighted the role of specific compounds and phenolic groups in antimutagenic activity of the respective teas. Further investigation into the phenolic composition of rooibos and the respective *Cyclopia spp.*, especially concerning the presence of non-

flavonoids and high molecular weight compounds such as proanthocyanidins, may provide insight into antimutagenic activity obtained with this study. It was also demonstrated that natural plant variation in rooibos and *Cyclopia spp.* within the same species, affects antimutagenic activity against AFB_1.

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Chapter 4

Effect of unfermented *Cyclopia spp.* (honeybush), *Aspalathus linearis* (rooibos) and *Camellia sinensis* (green tea) on rat hepatic cytochrome P450 stability and Type II substrate binding

Abstract

The effect of aqueous extracts from unfermented *Cyclopia spp.,* rooibos and green tea on cytochrome P450 stability in a liver homogenate (S9 fraction) prepared from Aroclor 1254 treated rats, and Type II substrate binding in the microsomal fraction, was investigated *in vitro.* Unfermented teas had a significant ($P < 0.05$) stabilising effect (measured as the % change in $\Delta A_{450–425}$) on cytochrome P450 in rat liver S9, demonstrated by less than 20% change in the presence of tea compared to *ca.* 78% for the control. Unfermented rooibos tea demonstrated the most stabilising (3.30%) of cytochrome P450 in rat liver S9, at a level similar ($P > 0.05$) to that of unfermented *C. subternata* and *C. sessiliflora*, while the stabilising effect of green tea (16.72%) was significantly ($P < 0.05$) less than rooibos. The decreased stability of cytochrome P450 (*ca.* 78%) was associated with substantial lipid peroxidation (1.760 nM MDA mg^{-1} protein) in rat liver S9. However, no correlation ($r = 0.018$, $P = 0.915$) existed for the effect of unfermented teas on cytochrome P450 stability with their inhibition of lipid peroxidation. This was attributed to differential effects noticed, i.e. green tea exhibited the strongest inhibition of lipid peroxidation, but demonstrated the least stabilising of cytochrome P450. This could be related to prooxidant activity of green tea at the high concentration levels used to investigate the stabilising effect on cytochrome P450. The inhibition of lipid peroxidation by rooibos was similar ($P > 0.05$) to unfermented *Cyclopia spp.,* except for *C. genistoides* exhibiting substantial less inhibition. The flavanol content of green tea showed a good marginal ($P < 0.1$) correlation ($r = 0.824$, $P = 0.086$) with the decrease in cytochrome P450 stability. A weak negative correlation ($r = -0.456$, $P = 0.026$) was observed for the flavonol/flavone content of unfermented *Cyclopia spp.* with protection against lipid peroxidation. The levels of aspalathin in rooibos and mangiferin in *Cyclopia spp.* did not correlate with the stabilising ability or protection against lipid peroxidation, by the extracts. Correlation of the stabilising effect on cytochrome P450 and the inhibition of lipid peroxidation by teas with their antimutagenic activity against metabolically activated aflatoxin B_1 (AFB_1) provided further insight into possible related mechanisms. Antimutagenic activity correlated weakly with a decreased stabilising effect of the teas on cytochrome P450 ($r =$

0.411, P = 0.013) and their protection against lipid peroxidation (r = 0.475, P = 0.003). The modulation of aniline-induced Type II binding to microsomal cytochrome P450 by green tea differed significantly from the modulation exhibited by rooibos and *Cyclopia spp.* Green tea resulted in an increased maximum absorbance in the difference spectra and decreased the minimum, while the inverse was observed for unfermented rooibos and unfermented *Cyclopia spp.* Of the selected phenolic compounds tested, the glycosides, aspalathin, mangiferin and hesperidin, demonstrated the least modulation of aniline-induced Type II binding to cytochrome P450.

Introduction

Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp.*) teas both contain a complex mixture of polyphenolic compounds (Rabe *et al*., 1994; Ferreira *et al*., 1995; Ferreira *et al*., 1998; Marais *et al*., 1998; Marais *et al*., 2000; Bramati *et al*., 2002; Kamara *et al*., 2003; Kamara *et al*., 2004) and possess antimutagenic (Standley, 1999; Marnewick *et al*., 2000; Richards 2002) and antioxidant (Von Gadow *et al*, 1997a; Von Gadow *et al*, 1997b; Hubbe & Joubert, 2000a; Hubbe & Joubert, 2000b, Richards, 2002) activity. Green tea produced from *Camellia sinensis* is a major source of natural polyphenols (Balentine *et al*., 1997; Chu, 1997; Wiseman *et al*., 1997; Beecher *et al.,* 1999; Gupta *et al*., 2002) and is well known for its antimutagenic (Kada *et al*., 1985; Mukhtar *et al*., 1992; Weisburger *et al*., 1996; Chen & Yen, 1997; Kuroda & Hara, 1999; Siess *et al*., 2000) and antioxidant (Ho *et al*., 1992; Yen & Chen, 1995; Cai *et al*., 2002; Atoui *et al*., 2004) activity.

Marnewick *et al*. (2000) proposed that one of the mechanisms responsible for antimutagenic activity of rooibos and honeybush (*Cyclopia intermedia*) teas could be due to interference of tea constituents with cytochrome P450 mediated metabolism of mutagens. Inhibition of the cytochrome P450 dependent bioactivation of promutagens have been reported as being the more imperative mechanism of protection against mutagenesis by green tea in the *Salmonella* mutagenicitiy assay (Bu-Abbas *et al.,* 1994; Bu-Abbas *et al*., 1997).

Cytochrome P450, a large family of hemoproteins, with the same ligand structure around the heme (Omura, 1993), is central to the detoxification of a remarkable number of foreign hydrophobic compounds including many therapeutic drugs and environmental pollutants (Kamataki, 1993; Omura, 1999). The detoxifying function of cytochrome P450 includes oxidative metabolism to render hydrophobic compounds more polar, as the first step of excretion from the body (Omura, 1999). Nevertheless, oxidation of some chemical compounds by

cytochrome P450 catalysed reactions produce highly reactive metabolites that are able to react with proteins and nucleic acids causing cytotoxicity and genotoxicity (Omura, 1999).

UV/visible spectrophotometry has been used extensively in detection and characterisation of cytochrome P450, owing to the distinct effect of substrate binding on the appearance of the overall spectrum, particularly with respect to the major absorption bands (Omura & Sato, 1962; Omura & Sato, 1964a; Schenkman *et al*., 1967; Schenkman *et al*., 1970; Schenkman *et al*., 1981).

Phenolic compounds from green tea result in a spectral change typical of Reverse Type I binding difference spectra, when added to rat hepatic microsomes from phenobarbital (PB)-treated rats, indicating interaction with cytochrome P450 (Wang *et al*., 1987). Addition of epigallocatechin gallate (EGCG) to dithionite reduced, and carbon monoxide-saturated rat liver microsomes increases absorption at 420 nm, while that at 450 nm is reduced, which is indicative of inhibition of carbon monoxide-reduced cytochrome P450 binding (Wang *et al*., 1987). At present no information is available on the interaction of *Cyclopia spp.* and rooibos tea polyphenols with cytochrome P450 *in vitro* and hence the effect of this interaction on the mutagenicity in experimental carcinogenesis.

Oxidation of unsaturated fatty acids of liver microsomal phospholipids results in destruction of the architecture of microsomal membranes (Vatsis *et al*., 1974) and hence could affect substrate enzyme interactions. It has been postulated that cytochrome P450 can be destroyed by $O_2^{\bullet}$ and H_2O_2, or via lipid peroxidation initiated by reactive oxygen species (Schaefer *et al*., 1985). The role of cytochrome P450 stability in the *Salmonella* mutagenicity assay on antimutagenic potency of phenolic compounds or different teas remains to be elucidated.

The present study investigated the effect of aqueous extracts of unfermented *Cyclopia spp.* (honeybush), unfermented *Aspalathus linearis* (rooibos) and *Camellia sinensis* (green) teas, as well as some of their phenolic constituents on the stability of cytochrome P450 in a rat liver homogenate fraction (S9) used in the *Salmonella* mutagenicity assay. The inhibition of lipid peroxidation in rat liver homogenate fractions, using malondialdehyde (MDA) as an index, by the aqueous tea extracts was also determined. Modulation of aniline-induced Type II binding to cytochrome P450 by the aqueous extracts of the different unfermented teas and selected phenolic compounds, was investigated utilising liver microsomes from Aroclor 1254 treated rats.

Materials and methods

Chemicals

Epigallocatechin gallate from green tea (> 95%), mangiferin from *Mangifera indica* leaves, hesperidin (97%), potassium chloride (KCl) (≥ 99.9%; ACS reagent), Tris [hydroxymethy] aminomethane (TRIZMA base) (≥ 99.9%), butylated hydroxytoluene (BHT; 2,[6]-di-tert-betyl-p-cresol) (≥ 99%), 2-thiobarbituric acid (TBA) (≥ 98%), trichloroacetic acid (TCA) (ACS reagent), aniline (glass distilled prior to use), bovine serum albumin (BSA) and Sepharose 2B were obtained from Sigma Chemicals Co. (St. Louis, USA). Sodium dithionite (≤ 85%) (sodium hydrosulphite; $Na_2S_2O_4 + H_2O$; assay idiometric) was supplied by BDH Chemicals Ltd. (Poole, England). Carbon monoxide (≥ 99.3%) was supplied by AFROX, BOC special products (Cape Town, SA) and dimethyl sulfoxide (DMSO for UV-spectroscopy) (≥ 99.8%) by Fluka/Sigma-Aldrich Chemie (Steinheim, Germany). Luteolin (> 90%), eriodictyol (HPLC-grade), hesperetin (≥ 95%) and chrysoeriol (HPLC-grade) were purchased from Extrasynthèse (Genay, France). BCA™ protein assay reagent A containing sodium carbonate, sodium bicarbonate, BCA™ detection reagent, and sodium tartrate in 0.1 N sodium hydroxide and BCA™ protein assay reagent B were purchased from Separations (Cape Town, SA). Aspalathin (≥ 95% as determined by HPLC and LC-MS) was isolated from unfermented rooibos tea at the PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis) unit of the Medical Research council (MRC, Bellville, SA) (Snijman *et al*., unpublished data). Solvents and chemicals used were analytical grade except if stated otherwise.

Preparation of samples

Plant material and preparation of extracts

Details of the plant material and preparation of the freeze-dried aqueous extracts (referred to as aqueous extracts) used in the present study are given in Chapter 3. Samples from six different batches of unfermented tea were included for each tea used in the investigation of cytochrome P450 stability and inhibition of lipid peroxidation. Three of the six samples of each tea were randomly selected for investigation of the modulation of cytochrome P450 substrate binding.

Preparation of liver homogenate fraction (S9) and microsomes

The liver homogenate fraction (S9) was prepared as described in Chapter 3. Microsomes were prepared by applying the S9 fraction to a Sepharose 2B column (2.8 x 29 cm, 1.69 mL per min)

with a 50 mM Tris-HCL buffer (pH 7.4) containing 150 mM KCl as eluent. Microsomes eluted in the void volume (15 mL) were collected and stored at -80°C. Microsomes and S9 were thawed at room temperature, on the day of the assay, and homogenised using 10 strokes in a glass tissue grinder with a loose pestle. The homogenised microsomes and S9 were kept on ice while the assay was performed.

Protein and cytochrome P450 determinations

The protein concentration of S9 homogenates and microsomes was determined according to the method described by Kaushal & Barnes (1986) with bovine serum albumin as standard and the cytochrome P450 content was assessed using the method of Omura & Sato (1964b).

The cytochrome P450 content of the S9 and microsomes was determined from the dithionite-reduced difference spectrum of carbon monoxide-saturated samples and expressed as nM mg^{-1} protein, using a millimolar extinction coefficient of 91 cm^{-1} mM^{-1} (Omura & Sato, 1964b). The S9 fraction was diluted to a concentration of 2 mg protein mL^{-1} and microsomes to 1 mg protein mL^{-1} with Tris-HCL buffer (50 mM, pH 7.4) containing 150 mM KCl (Tris-HCL buffer) and 5 mL pipetted into a glass vial. The samples were bubbled with CO for one min and then divided into two paired glass cuvettes (2.5 mL in each cuvette). The baseline was recorded (400–500 nm) before adding sodium dithionite to the sample cuvette. Difference spectra were recorded with a UVIKON 923 Double Beam UV/VIS Spectrophotometer (Analytical & Diagnostic Products, SA) using matched pair glass cuvettes (Lambda Instruments, SA) with a 1 cm path length. After subtracting the baseline, the cytochrome P450 content was calculated, using the following formula:

$$\text{nM P450 mg}^{-1}\text{ protein} = (A_{450}-A_{490})*1000/91*1/[\text{mg protein mL}^{-1}] \qquad \text{[equation 1]}$$

Stabilising of cytochrome P450 in the rat liver S9 fraction by aqueous extracts of the unfermented teas and selected phenolic compounds

The method used to assess the stabilising of cytochrome P450 in rat liver S9 by the different teas, were based on findings described in the method development in Addendum C. Difference spectra were recorded in duplicate for S9 samples containing 0.02% (w/v) aqueous extracts of the unfermented teas, i.e. *C. intermedia, C. subternata, C. genistoides, C. sessiliflora,* rooibos and green tea.

Stock solutions (0.2% w/v) were prepared by diluting the aqueous extracts in sterile distilled water. The S9 fraction was diluted to a protein concentration of 2 mg protein mL^{-1} with a Tris-HCL buffer (50 mM, pH 7.4). The reaction mixture consisted of 1 mL dissolved aqueous tea extract and 9 mL diluted S9 fraction. Controls contained 1.0 mL sterile distilled water instead of the aqueous tea extract. Difference spectra were recorded at 0 and 4 h, following the same procedure described for stability of cytochrome P450 in rat liver homogenate fractions in the method development section presented in Addendum C.

The stabilising effect of selected phenolic compounds on cytochrome P450 in the S9 fraction was determined in triplicate. Stock solutions (0.1 mM) were prepared in DMSO and 0.1 mL added to the S9 fraction (9.9 mL) to yield a concentration of 0.001 mM in the reaction mixture. Controls contained 0.1 mL DMSO instead of the phenolic compound. Difference spectra were recorded at 0 and 4 h, with a wavelength scan from 410-500 nm for samples containing phenolic compounds.

The stability of cytochrome P450 in the presence of the unfermented teas and selected phenolic compounds was calculated as the % change in $\Delta A_{450-425}$ using equations 2 and 3 (Addendum C). An increase in the % change in $\Delta A_{450-425}$ was representative of a decrease in the stability of cytochrome P450.

Assessment of lipid peroxidation in the rat liver S9 fraction

Stock solutions (0.001% w/v) of the aqueous tea extracts were prepared by dissolving in sterile distilled water. Stock solutions were diluted 10 times by addition of 1.5 mL to diluted S9 fraction (2 mg protein mL^{-1}) to obtain a concentration of 0.0001% in the reaction mixture (15 mL). Samples were incubated at 37˚C and 0 h samples (2 mL) collected after 15 min and stored on ice. After 4 hrs of incubation the remainder of each sample (2 mL) was collected and stored on ice. The assay was conducted in triplicate.

Lipid peroxidation in S9 fraction was determined by the formation of thiobarbituric acid reacting substances (TBARS) over a 4 h time period, measured as malondialdehyde (MDA), according to the method described by Beuge & Aust (1978) with the following modifications: Samples (2 mL) were mixed with 2 mL ice-cold TCA reagent (10%) containing BHT (12.5 µM) and EDTA (0.372 g EDTA L^{-1}). After centrifugation (Heraeus HS-F16/3 Megafuge 1.OR, Kendro Laboratory Products, Germany) at 2000 *x g* for 15 min at 4˚C, the supernatant (2 mL) was reacted with an equal volume of 0.67% (w/v) TBA solution, which was added to inhibit further oxidation. Samples were then incubated at 90˚C for 20 min and after cooling to room

temperature, absorbance was measured at 532 nm (Esterbauer & Cheeseman, 1990). Lipid peroxidation was calculated as nM MDA equivalents according to the following formula:

$$\text{MDA concentration (nM mg}^{-1}\text{ protein)} = \frac{A}{\varepsilon\, l}$$

A = Absorbance at 532 nm

ε = molar extinction coefficient of 1.56 x 105 M^{-1} cm^{-1} at 532 nm for MDA (Beuge & Aust, 1978).

l = path length of cell (1 cm)

Inhibition of lipid peroxidation was calculated as the difference of MDA formation in the control and the samples after 4 h at 37˚C, as a percentage of the MDA concentration in the control.

$$\text{\% Inhibition of lipid peroxidation} = \frac{[MDA_{\text{control (0 h - 4 h)}} - MDA_{\text{sample (0 h - 4 h)}}}{MDA_{\text{control (0 h - 4 h)}}]} \times 100$$

Modulation of the aniline Type II substrate binding to rat liver microsomal cytochrome P450 by aqueous extracts and selected phenolic compounds

The modulation of the aniline-induced Type II binding to cytochrome P450 by aqueous extracts at the same concentration and selected phenolic compounds was studied by a modified method described by Leibman *et al*. (1969).

Liver microsomes, prepared from the S9 fraction obtained from Aroclor 1254 treated rats, were diluted to a protein concentration of 1 mg mL^{-1} with a Tris-HCL buffer (50 mM, pH 7.4). Stock solutions of aqueous tea extracts (0.8% w/v), dissolved in sterile distilled water, and phenolic compounds (2.5 mM and 0.5 mM) dissolved in DMSO, were prepared and added in 0.1 mL quantities to microsomes to obtain a concentration of 0.016% (aqueous tea extracts) and 0.05 and 0.01 mM of the phenolic compounds in the reaction mixture (5 mL). For control measurements, aniline-induced substrate binding was monitored in the absence of the aqueous

tea extracts or phenolic compounds and carried out in the presence of equal amounts of sterile distilled water (control for tea) and DMSO (control for phenolic compounds).

Samples were incubated for 10 min at 20°C, where after the baseline was recorded. The substrate (aniline) was added in 3 µl quantities to the sample cell to yield a concentration of 1 mM, and an equal amount of buffer to the reference cell (Schenkman *et al*., 1970). Subsequent to proper mixing, the difference spectra were recorded (350–500 nm). The binding spectra of aniline were determined by subtracting the baseline. The reference and sample cuvettes both contained microsomes and tea extracts or phenolic compounds when the baseline was recorded, thus correcting for any effect of the colour of the tea or phenolic compounds. The maximal spectral interaction (ΔA_{max}) value was calculated according to equation 5.

ΔA_{max} = maximum absorption between 390-400nm – minimum absorption between 425-430 nm [equation 5]

The change that occurred in the ΔA_{max} in the presence of teas and phenolic compounds were calculated by the % change in ΔA_{max} using the following formula:

$$\% \text{ Change in } \Delta A_{max} = [(\Delta A_{max(control)} - \Delta A_{max(sample)}) / \Delta A_{max(control)}] * 100 \quad \text{[equation 6]}$$

Dose response studies were performed for *C. intermedia*, rooibos and green tea by preparing stock solutions of 0.8% (w/v) and subsequent dilutions.

Statistical Analysis

Repeated measures analysis of variance (ANOVA) (Statistica Version 6.1) was performed to determine the difference in the stabilising effect of the teas and phenolic compounds on cytochrome P450 in S9. One-way analysis of variance (ANOVA) (Statistica Version 6.1) was performed to determine the difference between the interference of the different aqueous tea extracts and phenolic compounds with rat liver microsomes. A Bonferroni Post HOC test was utilised to compare means when a significant difference was evident.

Statistical analyses of inhibition of lipid peroxidation was performed with SAS version 9.1, and included tests for normality among the groups with the Kolmogorov-Smirnof Test, and testing for homogeneity of group variances with Levene's Test. A one-way analysis of variance (ANOVA) was performed for testing significant group differences, followed by a post-HOC

Tukey's Studentised Range test to determine which groups differed significantly. Means were correlated with the Pearson's correlation coefficient. A significance level of 5% ($P < 0.05$) was used as guideline for determining significant differences.

Results

Stabilising of cytochrome P450 in rat liver S9 by aqueous extracts prepared from unfermented teas and selected phenolic compounds

Aqueous extracts from unfermented *Cyclopia spp.,* rooibos and green tea demonstrated a stabilising effect on cytochrome P450. This was evident from the considerable reduction of the change that occurs in difference spectra of CO saturated, dithionite reduced rat liver S9 over 4 hrs at 37ºC in the control, as illustrated in Fig. 1.

It was established with preliminary studies (Addendum C) that an increase in the % change in $\Delta A_{450-425}$ is associated with a decrease in the cytochrome P450 content and thus the stability of the enzyme in the rat liver S9 fraction. The % change in $\Delta A_{450-425}$ in samples containing 0.02% (w/v) of the aqueous extracts, were less than 20% for all the teas, as opposed to the 77.67% change that occurred in the absence of tea (control) (Table 1).

Unfermented rooibos tea (3.30%) and unfermented *C. subternata* (7.57%) and *C. genistoides* (8.46%) demonstrated significantly ($P < 0.05$) better stabilising of cytochrome P450 than green tea (16.72%). The stabilising effect of unfermented *C. intermedia* (10.76%) and *C. sessiliflora* (11.49%) was similar ($P > 0.05$).

Differences in the ability of selected phenolic compounds to counteract the % change in $\Delta A_{450-425}$ at a concentration level of 0.001 mM was evident in the S9 fraction after 4 hrs as demonstrated in the difference spectra illustrated in Fig. 2. Hesperidin had no stabilising effect since the % change was similar ($P > 0.05$) to that occurring in the control, while all the other phenolic compounds demonstrated significant stabilising ($P < 0.05$) (Table 2). Aspalathin demonstrated relatively weak stabilising resulting in a relatively high % change at 49.54%, comparable ($P > 0.05$) to the 39.46% change in samples containing mangiferin. Eriodictyol resulted in markedly ($P > 0.05$) better stabilising of cytochrome P450 (29.39%) than mangiferin. The least change occurred in samples containing, EGCG (15.74%) and luteolin (17.46%), which had similar ($P > 0.05$) effects to chrysoeriol (23.87%), but significantly better stabilising ($P < 0.05$) than mangiferin. Although EGCG effectively inhibited the change in

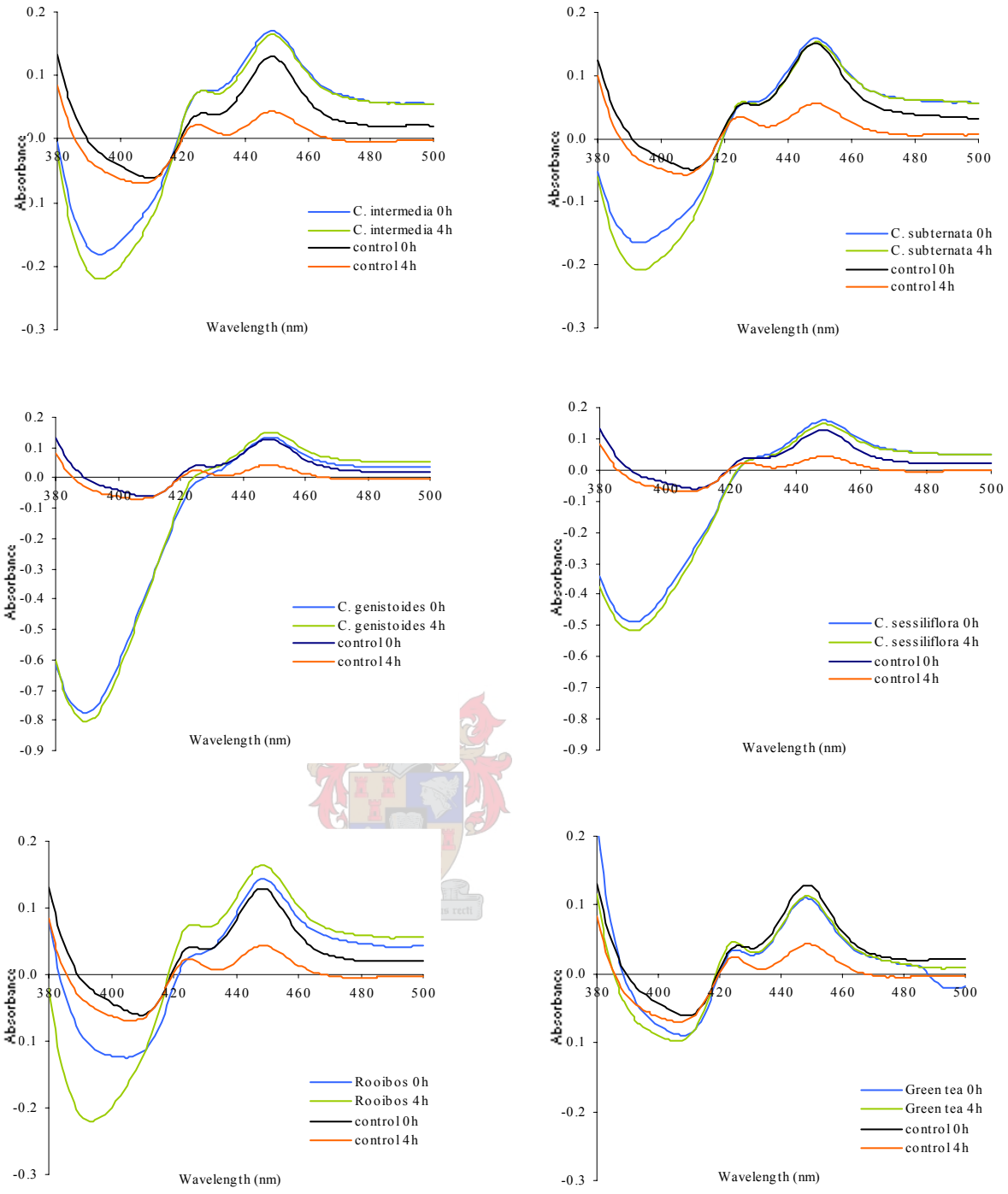


Figure 1 Difference spectra of the CO-saturated, dithionite reduced rat liver S9 containing aqueous tea extracts (0.02% w/v) from unfermented teas and controls, in demonstration of the stabilising of cytochrome P450 by different teas.

Table 1 The % change in $\Delta A_{450-425}$ in the difference spectra of the rat liver S9 fraction (2 mg mL^{-1} protein, 0.514 nM P450 mg^{-1} protein) containing 0.02% (w/v) aqueous extracts prepared from unfermented tea.

Sample	% Change in $\Delta A_{450-425}$ [a]
Control	77.67 ± 3.63 a
C. intermedia	10.76 ± 4.06 bc
C. subternata	7.57 ± 5.07 c
C. genistoides	8.46 ± 4.86 c
C. sessiliflora	11.49 ± 5.24 bc
Rooibos tea	3.30 ± 3.12 c
Green tea	16.72 ± 5.91 b

[a] Values represent the means ± SD of the % change in $\Delta A_{450-425}$ for six samples (n = 6) of the unfermented teas determined in duplicate. Means followed by the same letter are not significantly different (P > 0.05).

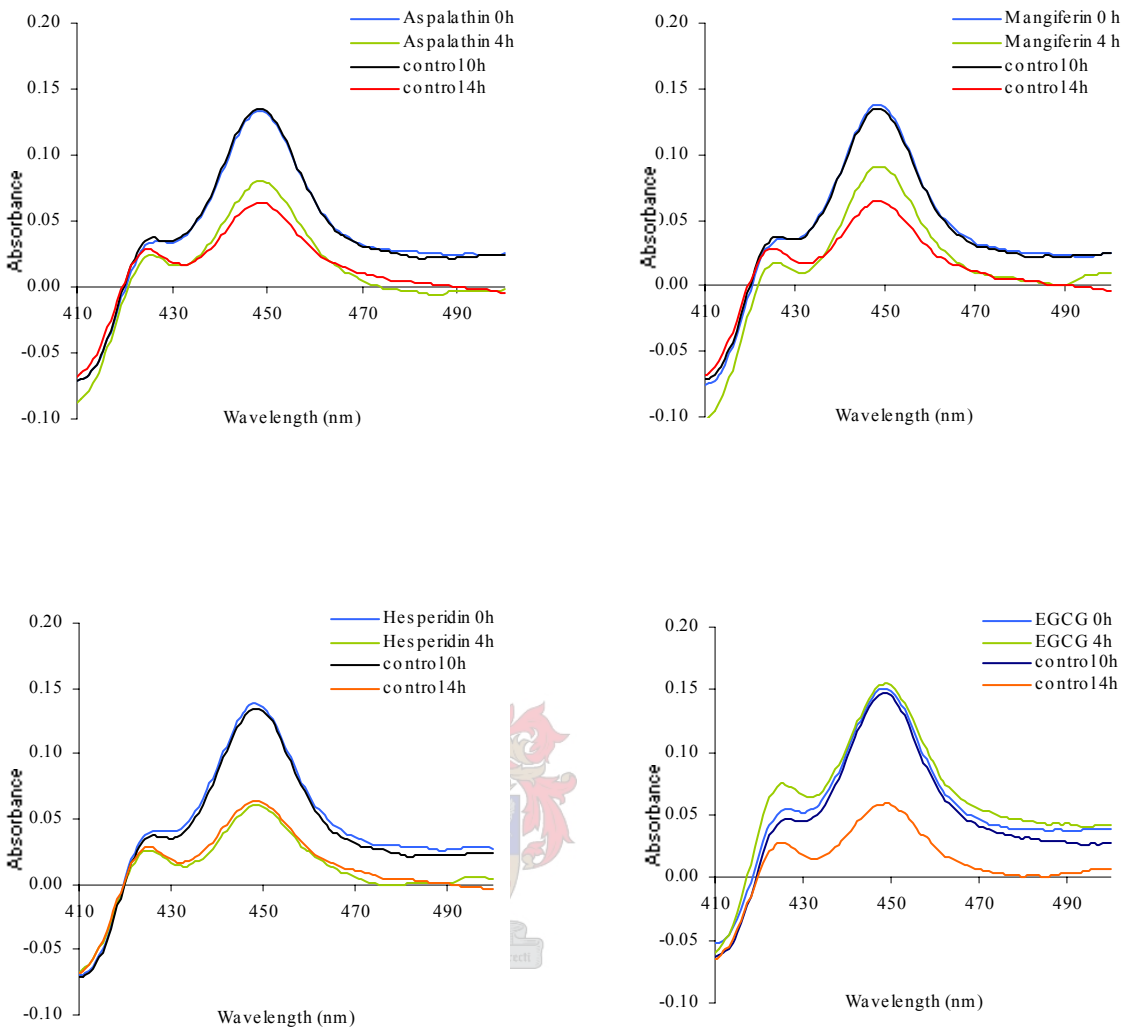
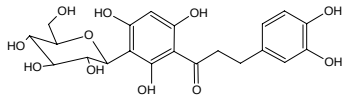
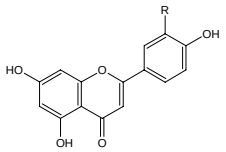
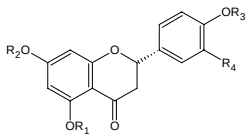
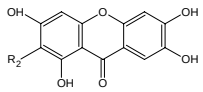
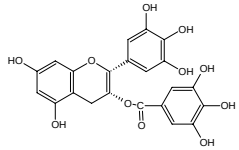


Figure 2 Difference spectra recorded of the CO-saturated, dithionite reduced rat liver S9 containing aspalathin, mangiferin, hesperidin and EGCG at a concentration level 0.001 mM and controls, in demonstration of the stabilising of cytochrome P450 by selected phenolic compounds.

Table 2 The % change in $\Delta A_{450-425}$ in the difference spectra of rat liver S9 fraction (2 mg mL^{-1} protein, 0.514 nM P450 mg^{-1} protein) containing selected phenolic compounds at a concentration level of 0.001 mM.

Compound type	Structure	Compound	Substitution	% Change in $\Delta A_{450-425}$[a]
Control				66.15 ± 4.85 a
Dihydro-chalcone		Aspalathin		49.54 ± 4.62 b
Flavone		Luteolin	R=OH	17.64 ± 4.97 de
		Chrysoeriol	R=OMe	23.87 ± 6.13 de
Flavanone		Eriodictyol	R_1=R_2=R_3=H, R_4=OH	29.39 ± 4.26 cd
		Hesperidin	R_1=H, R_2=rutinosyl, R_3=Me, R_4=OH	65.99 ± 5.43 a
Xanthone		Mangiferin	R_2=2-β-*D*-glucopyranosyl	39.46 ± 7.01 bc
Catechin		EGCG		15.74 ± 3.49 e

[a] Values represent the means ± SD of the % change in $\Delta A_{450-425}$ determined in triplicate. Means followed by the same letter are not significantly different (P > 0.05).

$\Delta A_{450-425}$ over 4 hrs, an increase in the absorbance at 425 nm, which was not present for any of the other compounds, was evident from the difference spectra (Fig. 2).

Inhibition of lipid peroxidation in the rat liver S9 fraction

Incubation of the S9 fraction (2 mg protein mL^{-1}) at 37ºC for 4 hrs resulted in a significant increase in lipid peroxidation (1.760 nM MDA mg^{-1} protein). The unfermented teas exhibited considerable inhibition of lipid peroxidation, demonstrated by the lower levels of MDA formed in the presence of tea. Green tea exhibited almost complete inhibition of lipid peroxidation (99.30%) at a concentration level of 0.0001% (w/v) (Fig. 3). The protection afforded by unfermented rooibos and *Cyclopia spp.*, except *C. genistoides,* (between 75.46-86.92% at 0.0001 (w/v)) was similar ($P > 0.05$) to that of green tea (83.47%) at 0.00005% (w/v). Unfermented *C. genistoides* demonstrated significantly ($P < 0.05$) less protection and afforded only 46.99% inhibition of lipid peroxidation in the S9 fraction.

Significant differences ($P < 0.05$) in the inhibition of lipid peroxidation by the individual samples from the six different batches for each tea were demonstrated and is presented in Table 3. The most variation was evident for unfermented *C. subternata* ranging between 33.77% to 98.55% (Table 3). Inhibition of lipid peroxidation also varied considerably for the other unfermented *Cyclopia spp.*, while less variation was observed for unfermented rooibos and green tea (Table 3).

Modulation of the aniline Type II substrate binding to rat liver microsomal cytochrome P450 by aqueous extracts and selected phenolic compounds

The dose dependent modulation of aniline-induced (1 mM) type II binding of cytochrome P450 in rat liver microsomes, measured as the % change in the maximal spectral interaction (ΔA_{max}) in the difference spectra, by aqueous extracts from unfermented *Cyclopia spp.*, unfermented rooibos and green tea is illustrated in Fig. 4. The three concentration levels tested for unfermented *C. intermedia* did not differ ($P > 0.05$) in terms of the modulating effect and resulted in less than 10% change in ΔA_{max} (Fig. 4a). A typical dose response effect was obtained when using four concentration levels of rooibos tea (Fig. 4b). The highest concentration level (0.016% w/v) resulted in a significantly ($P < 0.05$) higher % change in ΔA_{max}, while the change with 0.002% was significantly less than with concentration levels of 0.004 and 0.008% (w/v).

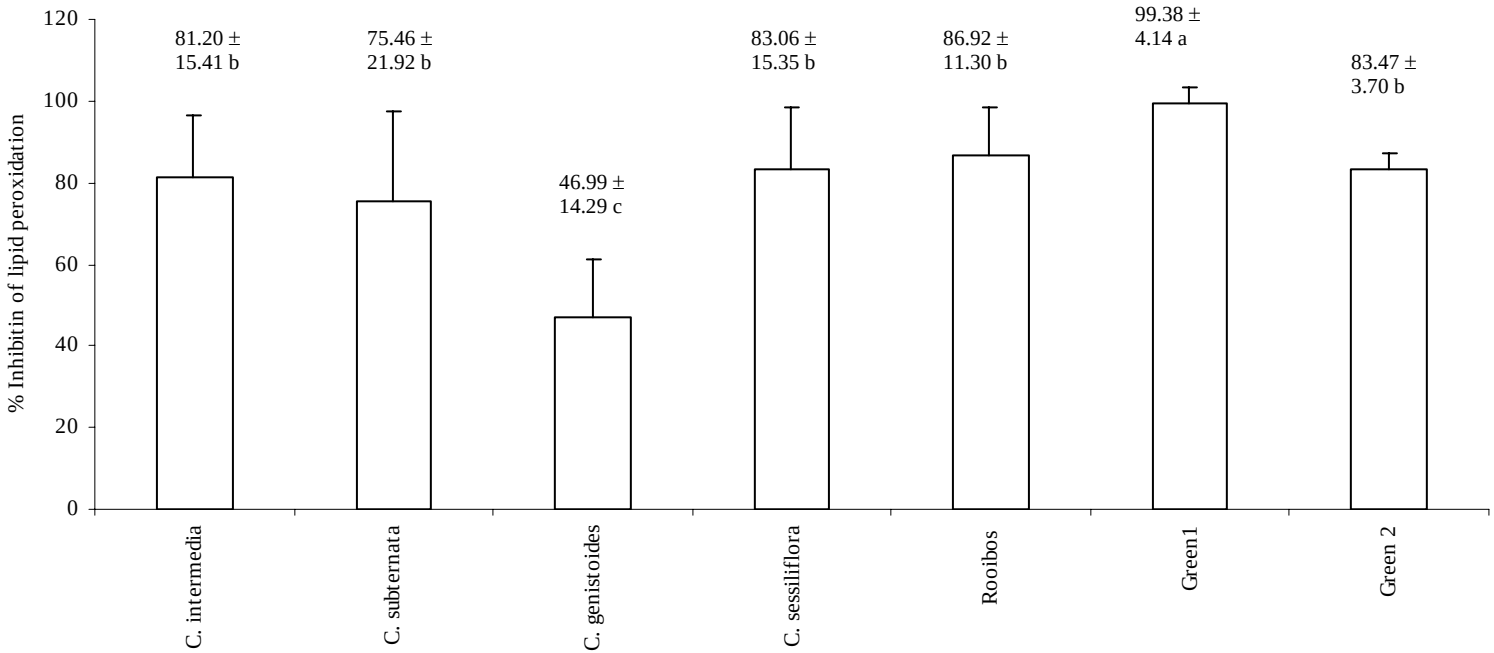


Figure 3 Mean inhibition of lipid peroxidation in the S9 fraction by aqueous extracts prepared from unfermented tea (0.0001 % w/v) determined for six different batches (n = 6) of plant material for each tea (triplicate analysis). Inhibition of lipid peroxidation was determined at two concentrations for green tea, i.e. green 1 (0.0001%) and green 2 (0.00005%). Values represent the means ± SD (indicated by error bars). Means followed by the same letter did not differ significantly ($P > 0.05$).

Table 3 Inhibition of lipid peroxidation in the S9 fraction over 4 hrs at 37°C by different samples from six batches of unfermented *Cyclopia spp.*, rooibos and green tea.

	Batches[a]					
Tea	**1**	**2**	**3**	**4**	**5**	**6**
C. intermedia,uf	82.23 ± 1.66 bc	92.76 ± 4.86 ab	84.81 ± 3.73bc	51.54 ± 7.16d	79.16 ± 3.99c	96.70 ± 2.87a
C. subternata,uf	74.84 ± 4.47 b	79.27 ± 1.26 b	98.55 ± 8.04 a	71.90 ± 1.98 b	33.77± 2.32 c	94.45± 0.75 a
C. genistoides,uf	45.24 ± 4.26 bc	56.12 ± 2.28 ab	42.30 ± 4.43 bc	36.89 ± 8.47 c	30.87± 9.28 c	70.50± 0.29 a
C. sessiliflora, uf	69.21 ± 3.91 c	98.42 ± 2.03 a	91.37 ± 2.85 ab	86.13 ± 2.35 b	58.22± 8.34 c	95.04± 0.83 ab
Rooibos tea, uf	74.69 ± 4.68 b	97.02 ± 1.70 a	89.06 ± 1.09 a	70.99 ± 4.98 b	92.94± 7.73 a	96.82± 2.87 a
Green tea 1[b]	98.66 ±1.51 ab	92.74 ± 0.94 b	100.14 ± 2.82 a	98.91 ± 1.40 ab	104.12± 4.56 a	101.70± 1.48 a
Green tea 2[b]	82.39± 0.90 ab	81.10± 0.86 ab	86.06± 5.67 ab	79.12± 1.68 b	85.08± 2.39 ab	87.07± 1.41 a

[a] Values represent the means ± SD of the % inhibition of lipid peroxidation (triplicate analysis). Means in the same row followed by the same letter are not significantly different (P > 0.05).

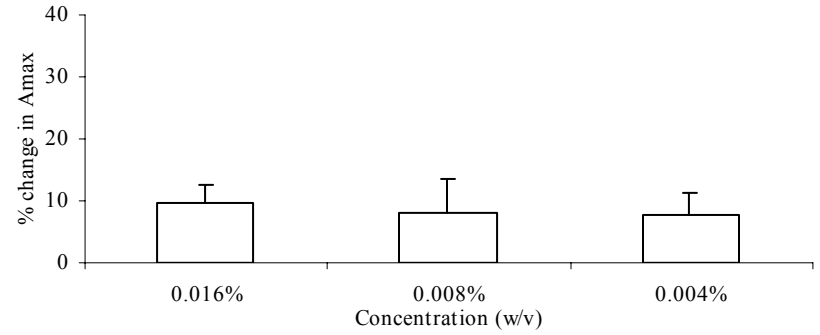
[b] Inhibition of lipid peroxidation was determined at two concentrations for green tea i.e. green 1 (0.0001%) and green 2 (0.00005%).

The effect of green tea on aniline-induced Type II binding to cytochrome P450 was the opposite to that of unfermented rooibos and *Cyclopia spp.*, in that green tea resulted in a decrease in ΔA_{max}, reflected by the negative value for the % change in $\Delta A_{max.}$ Concentration levels of 0.016 and 0.004% resulted in significantly higher negative changes than at 0.001% (Fig. 4c). Contrasting to the increase at the other concentration levels, a decrease in ΔA_{max} was observed, but only of 2.92 % change in ΔA_{max} at the very low level of 0.00025% (w/v).

The modulation of aniline (1 mM) binding to cytochrome P450 by aqueous extracts of unfermented teas was compared at a concentration level of 0.016% (w/v) with the difference spectra depicted in Fig. 5 and the effects summarised in Fig. 6. Unfermented rooibos tea, *C. subternata, C. genistoides* and *C. sessiliflora* had a more pronounced effect on the binding of aniline to cytochrome P450 at the concentration level tested. *Cyclopia intermedia* was less effective than rooibos tea and *C. sessiliflora*, while ΔA_{max} was increased upon the addition of green tea resulting in a negative % change in ΔA_{max} of 15.47%.

The modulation of aniline-induced Type II substrate binding of cytochrome P450 in rat liver microsomes by selected phenolic compounds was also compared in terms of the % change in ΔA_{max} at concentration levels of 0.01 and 0.05 mM. The changes relative to that of the control are depicted in Fig. 7 and summarised in Fig. 8. No significant dose response effect was observed, although the modulation at 0.01 mM was marginally ($P < 0.1$) lower than that observed at 0.05 mM for all the compounds. Aspalathin, hesperidin, mangiferin and EGCG demonstrated the least modulation of binding at both concentration levels, also clearly illustrated in Fig. 7 by the difference spectra. The highest modulation of Type II binding was demonstrated by hesperetin, resulting in 48.90% change in ΔA_{max} at a concentration level of 0.05 mM and 40.7% at 0.01 mM (Fig. 8). Luteolin and chrysoeriol resulted in markedly higher, but not significant ($P > 0.05$), changes in the difference spectra than eriodictyol at 0.05 mM. The effect of eriodictyol at 0.01 M was, however, significantly less than that of chrysoeriol, but not than that of luteolin (Fig. 8). Of interest was the shift in the minimum band of the aniline-induced spectrum from 390 to 400 nm in the presence of luteolin, hesperitin (Fig. 7), eriodictyol and chrysoeriol. No shift was noticed upon the addition of aqueous extracts and aspalathin, mangiferin, hesperidin and EGCG (Fig. 7).

a)



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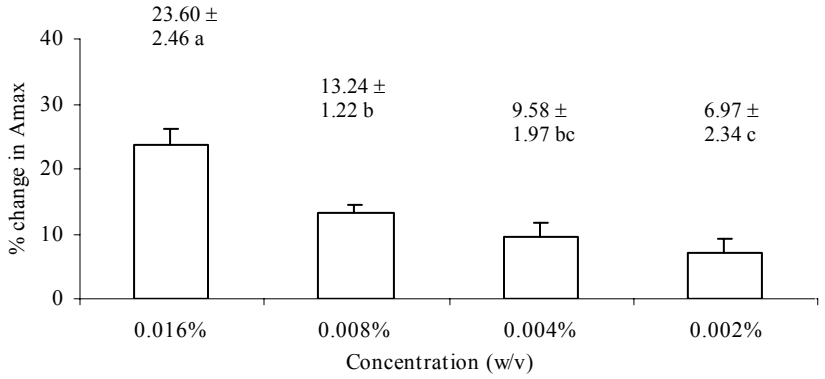

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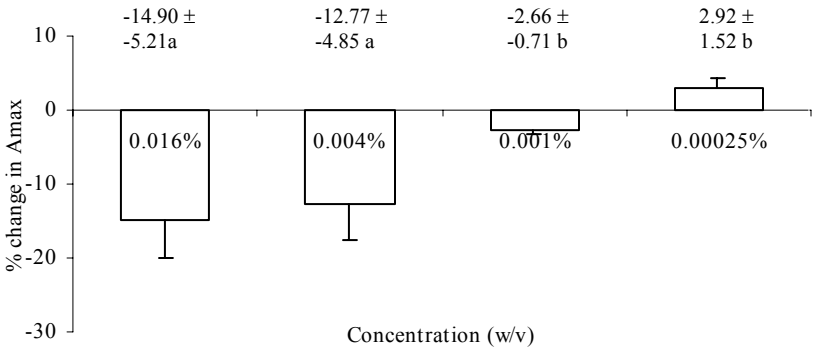


Figure 4 Dose dependant modulation of the aniline-induced Type II substrate binding to rat liver microsomal cytochrome P450 of aqueous extracts prepared from a) unfermented *C. intermedia,* b) unfermented rooibos and c) green tea. Values are the means ± S.D. (indicated by error bars) of samples from three different batches (n = 3). Means followed by the same letter did not differ significantly (P > 0.05).

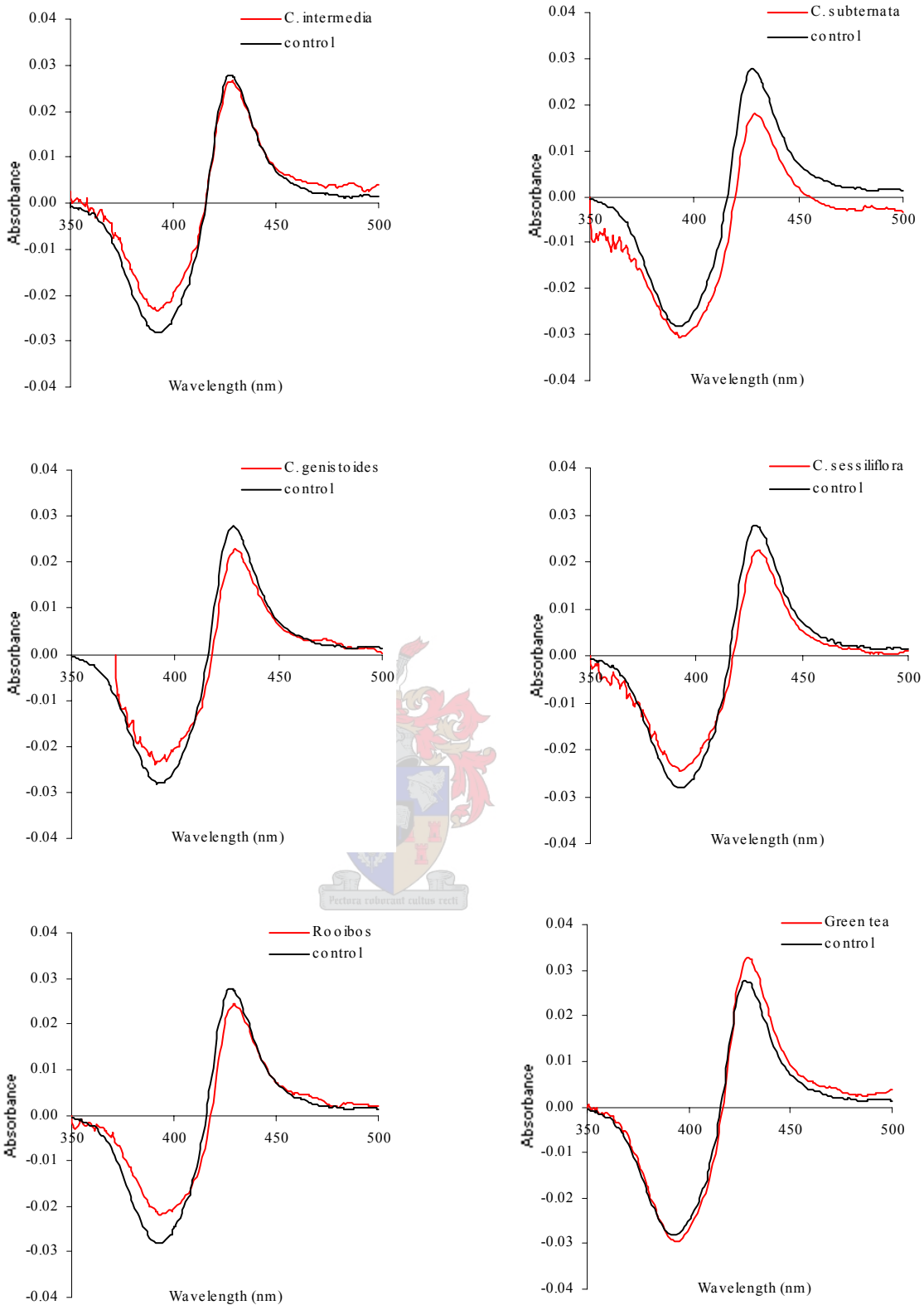


Figure 5 Modulation of the aniline-induced Type II substrate binding to rat liver microsomal cytochrome P450 of aqueous extracts (0.016% w/v) prepared from unfermented *Cyclopia spp.*, rooibos and green tea (graphs are the averages of triplicate measurements).

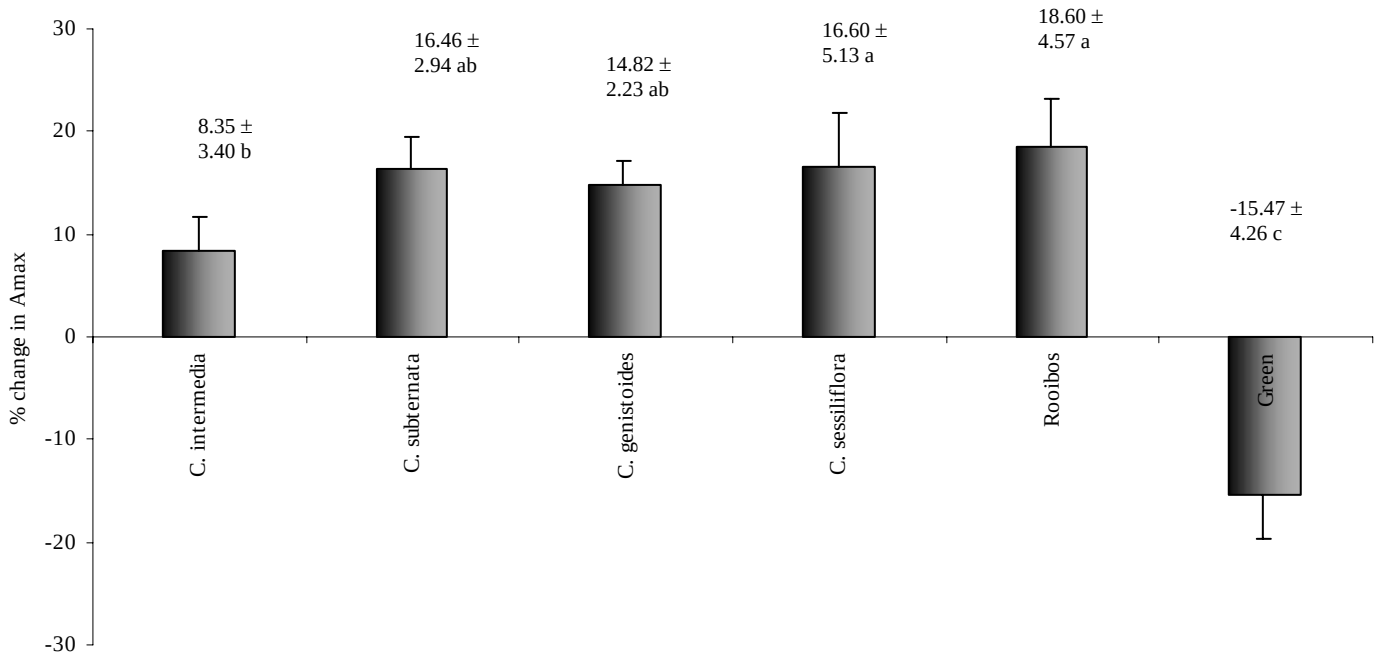


Figure 6 Modulation of the aniline-induced Type II substrate binding to rat liver microsomal cytochrome P450 of aqueous extracts prepared from unfermented *Cyclopia spp.* rooibos and green tea at a concentration level of 0.016% (w/v). Values are the means ± S.D. (indicated by error bars) of samples from three different batches (n = 3). Means followed by the same letter did not differ significantly (P > 0.05).

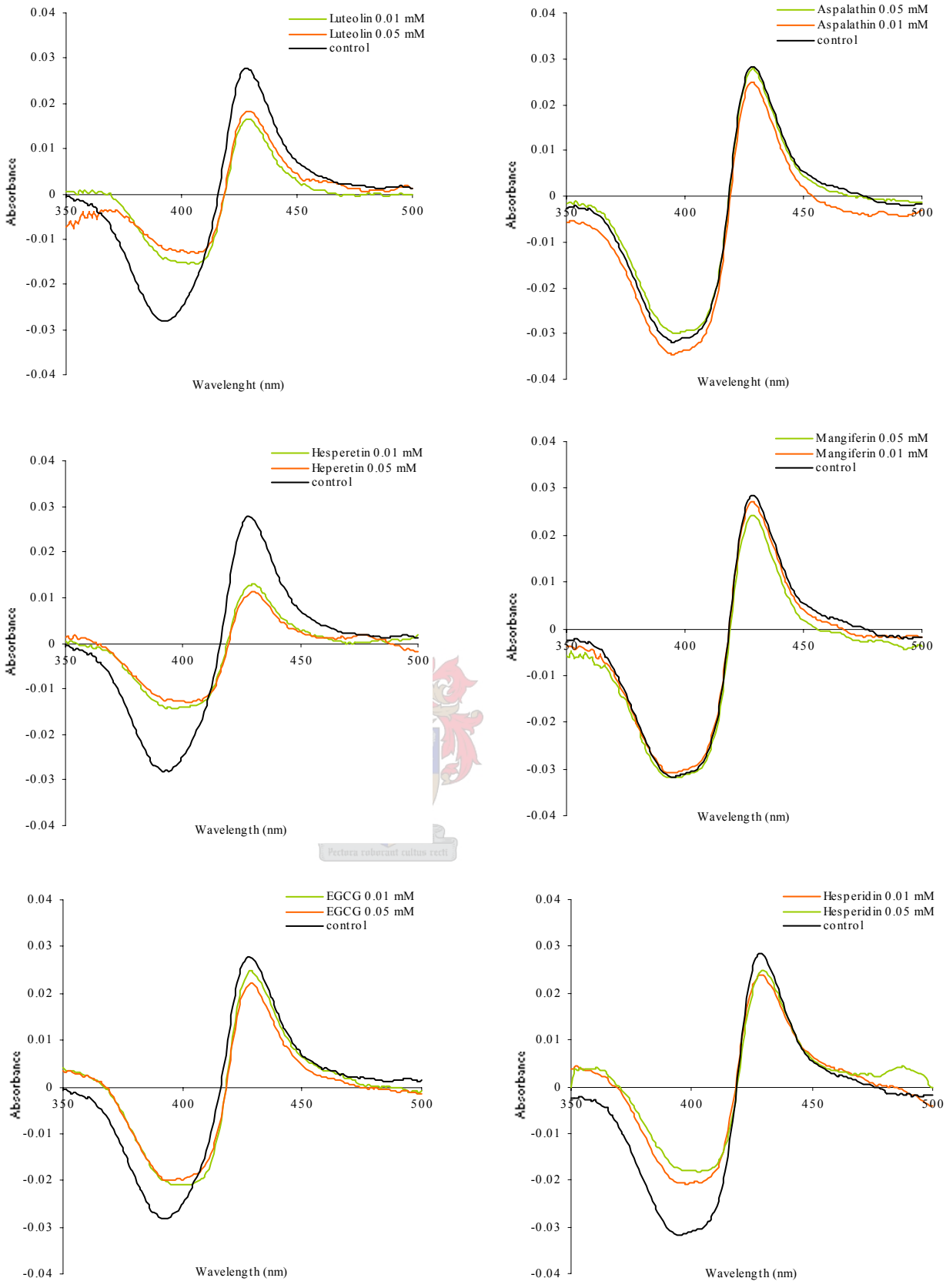


Figure 7 Modulation of aniline-induced Type II binding to cytochrome P450 in rat liver microsomes by selected phenolic compounds at a concentration level of 0.01 and 0.05 mM (graphs are the averages of triplicate measurements).

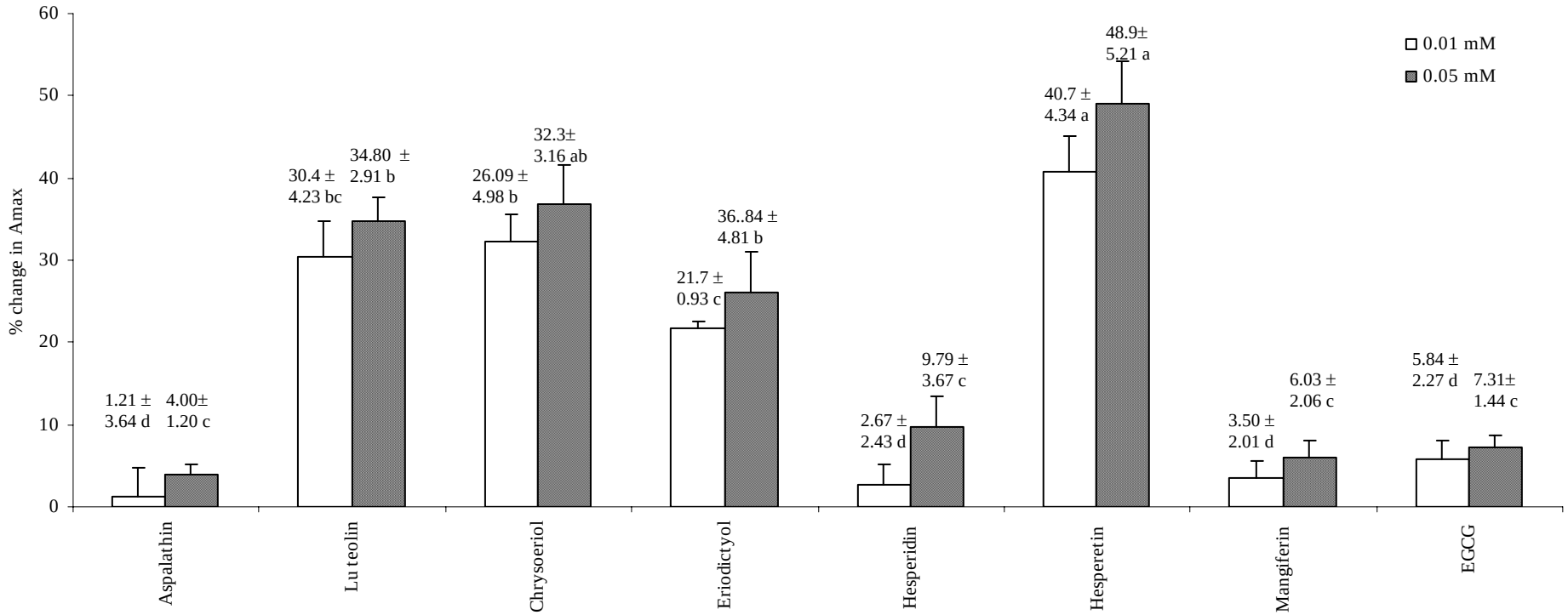


Figure 8 Modulation of aniline-induced Type II binding to cytochrome P450 by selected phenolic compounds at concentration levels of 0.01 mM and 0.05 mM. Values are the means ± SD (indicated by error bars) of the % change in ΔA_{max}. Means at the same concentration level followed by different letters differed significantly ($P < 0.05$).

The concentration levels of the aqueous extracts of the teas in the *Salmonella* mutagenicity assay and for the studies of cytochrome P450 stability and modulation as well as the concentrations used to investigate protection against lipid peroxidation, were calculated for comparative purposes and are summarised in Table 4. The concentration of aqueous extracts used in the different assays were similar, except for lipid peroxidation where the level of tea extracts comprised 0.001 g mL^{-1}.

Correlation of the antimutagenic activity of unfermented teas with the stabilising of cytochrome P450 and inhibition of lipid peroxidation in rat liver S9

The stabilising effect of unfermented teas on cytochrome P450 and prevention of lipid peroxidation, in rat liver S9 were compared to the antimutagenic activity assessed in Chapter 3 to determine if any relationship exist (Fig. 9).

The % change in $\Delta A_{450-425}$ in rat liver S9 that occurred in the presence of unfermented tea extracts correlated weakly with the antimutagenic activity of the tea samples against AFB_1 ($r = 0.411$, $P = 0.013$) (Fig. 9a), while no correlation ($r = 0.018$, $P = 0.915$) was evident with the protection against lipid peroxidation demonstrated by the tea extracts (Fig. 9b). A weak correlation ($r = 0.475$, $P = 0.003$) was observed for the ability of teas to inhibit lipid peroxidation in the S9 fraction and inhibition of AFB_1- induced mutagenesis (Fig. 9c).

Correlations of effects of phenolic composition of teas with stabilising of cytochrome P450 and prevention of lipid peroxidation

The potential of different tea extracts to stabilise cytochrome P450, measured as the % change in $\Delta A_{450-425}$ in the presence of tea, and the inhibition of lipid peroxidation was correlated with the composition data of the tea extracts. This was aimed at determining the role of phenolic groups (total polyphenols, flavanols and flavonol/flavones) and individual compounds, aspalathin, orientin and iso-orientin in unfermented rooibos, and mangiferin and hesperidin in *Cyclopia spp*, in the activity in the repective assays.

The overall correlation of the % change in $\Delta A_{450-425}$ and inhibition of lipid peroxidation with the total polyphenol, flavanol and flavonol/flavone contents of aqueous extracts is summarised in Table 5. The % change in $\Delta A_{450-425}$ did not correlate ($r = -0.047$, $P = 0.790$) with the total polyphenol content of the teas.

Table 4 Concentration levels of aqueous extracts from *Cyclopia spp.*, rooibos and *Camellia sinenesis* teas and selected phenolic compound as well as the protein concentration in different assays for comparative purposes

	Aqueous extract **mg mL^{-1}**	**Phenolic compound** **mM mL^{-1}**	**Protein** **mg mL^{-1}**
Salmonella mutagenicity assay	0.179	0.107[a] 0.036[b]	0.286
Stability studies	0.200	0.001	1.800[c] 1.980[d]
Binding studies	0.160	0.050[e] 0.010[f]	0.980
Lipid peroxidation	0.001		1.808

[a] Concentration of selected phenolic compounds in the *Salmonella* mutagenicity assay at a concentration level of 0.03 mM per plate.

[b] Concentration of selected phenolic compounds in the *Salmonella* mutagenicity assay at a concentration level of 0.01 mM per plate.

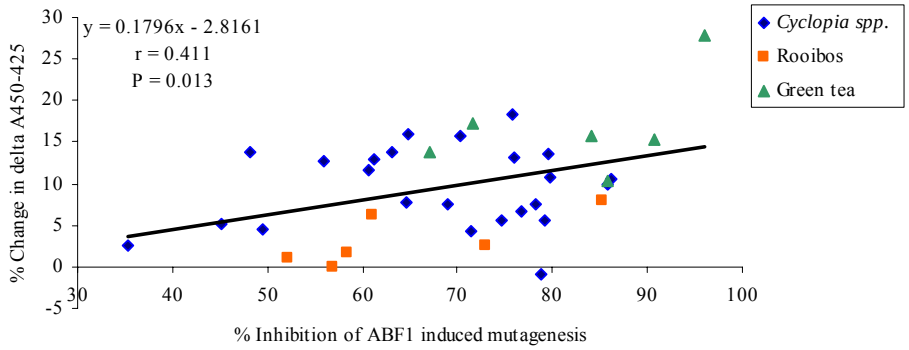
[c] Protein concentration in the stability studies with aqueous tea extracts.

[d] Protein concentration in the stability studies with selected phenolic compounds.

[e] Concentration of selected phenolic compounds in the binding studies at a concentration level of 0.05 mM.

[f] Concentration of selected phenolic compounds in the binding studies at a concentration level of 0.01 mM.

a)



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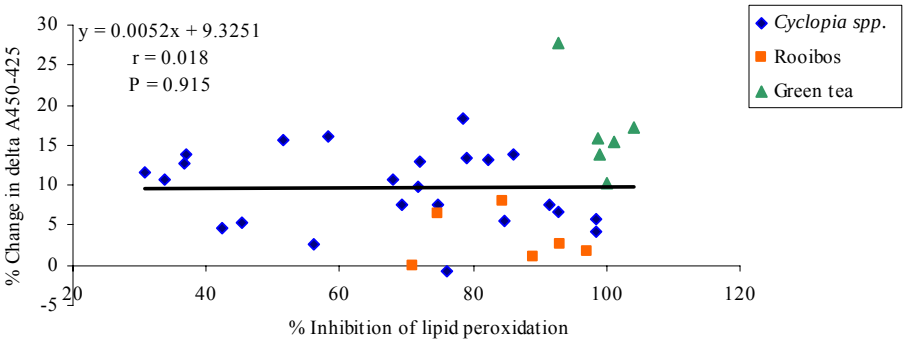

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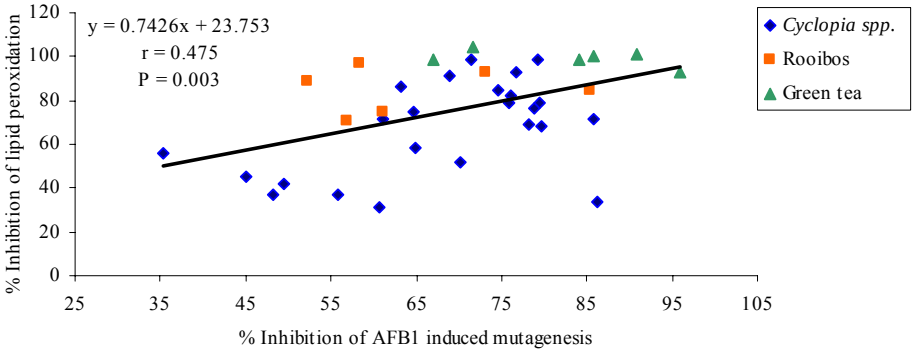


Figure 9 Correlation of the % change in $\Delta A_{450-425}$ in the presence of the unfermented teas with a) their antimutagenic activity against AFB_1 and b) their protection against lipid peroxidation in S9. The correlation of c) the protection against lipid peroxidation in S9 by the teas with their antimutagenic activity against AFB_1.

A moderate (r = 0.526, P = 0.001) to weak (r = 0.486, P = 0.003) correlation was observed for the flavanol content of the aqueous extracts with the % change in $\Delta A_{450-425}$ and inhibition of lipid peroxidation, respectively (Figs. 10a and b).

These correlations are, however, partially attributed to sample distribution, caused by the high flavanol content of green teas, which resulted in two separate clusters of data points. Protection against lipid peroxidation correlated weakly (r = 0.437, P = 0.009) with the total polyphenol content of the unfermented teas (Fig. 10c). In the latter case the data points were of more even distribution. A weak negative correlation (r = -0.447, P = 0.007) was observed for the protection of aqueous extracts against lipid peroxidation with flavonol/flavone content, while an even weaker negative correlation (r = -0.352, P = 0.030) was evident for the % change in $\Delta A_{450-425}$ with the flavonol/flavone content of aqueous extracts.

Correlations for the respective teas, with the different phenolic groups in unfermented *Cyclopia spp,* rooibos and green tea is summarised in Table 6. Only the flavonol/flavone content of *Cyclopia spp.* correlated (r = -0.456, P = 0.026) with the protection against lipid peroxidation (Fig. 11a). No significant (P < 0.05) correlations were observed for the levels of phenolic groups present in unfermented rooibos with the activity observed in both assays. Stability gave a strong but marginal (P < 0.1) correlation (r = 0.824, P = 0.086) with the flavanol content of green tea (Fig. 11b). Only a few data points were used to calculated the correlation, but a clear trend is evident from sample distribution illustrated in Fig. 11b. The total polyphenol content of green tea had a negative correlation (r = - 0.837), which was marginally (P < 0.1) significant (P = 0.077) with the protection against lipid peroxidation. The distribution of data points illustrated in Fig. 11c, however, indicates that no trend exist and the correlation appear to be a result of sample distribution.

Correlation of the % change in $\Delta A_{450-425}$ (stability) and the inhibition of lipid peroxidation in the S9 fraction by unfermented teas with the aspalathin, orientin and iso-orientin content of rooibos and the mangiferin and hesperidin contents of *Cyclopia spp.* is summarised in Table 7. None of the rooibos flavonoids correlated with activity of aqueous extracts in either of the assays. The mangiferin content of *Cyclopia spp.* did not correlate (r = -0.054, P = 0.802) with the % change in $\Delta A_{450-425}$, but distinct clustering of *C. genistoides*, attributed to higher mangiferin content is illustrated in Fig 12a. The negative correlations obtained for the mangiferin content of *Cyclopia spp.* with the inhibition of lipid peroxidation also appear to be the result of the clustering of data (Fig. 12b).

Table 5 Correlation of overall stabilising ability[a] and inhibition of lipid peroxidation[b] with the content of phenolic groups (total polyphenols, flavanols and flavonol/flavones) in aqueous extracts from unfermented *Cyclopia spp.*, rooibos and green tea.

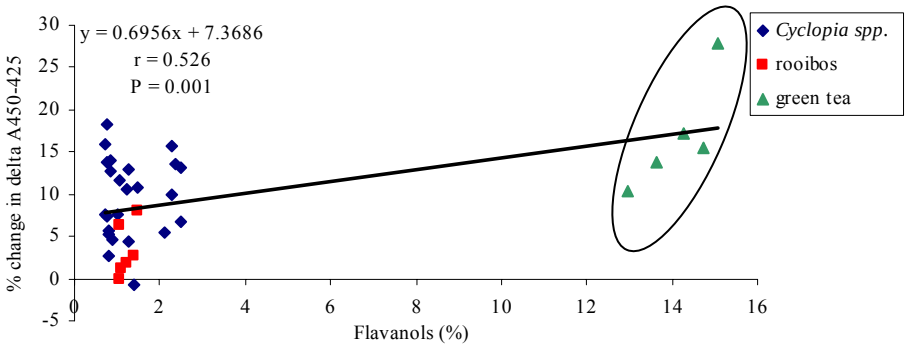
Assay	Total Polyphenols (g gallic acid equivalents $100\ g^{-1}$)	Flavanols (g catechin equivalents $100\ g^{-1}$)	Flavonol/Flavones (g quercetin equivalents $100\ g^{-1}$)
Stability (% change in $\Delta A_{450-425}$)	r = -0.047[c] (P = 0.790)	**r = 0.526 (P = 0.001)**	**r = -0.352 (P = 0.030)**
% Inhibition of lipid peroxidation	**r = 0.437 (P = 0.009)**	**r = 0.486 (P = 0.003)**	**r = -0.447 (P = 0.007)**

[a] Stability of cytochrome P450 in rat liver S9 measured as the % change in $\Delta A_{450-425}$ in the presence of unfermented *Cyclopia spp.* and unfermented rooibos tea obtained from six different batches (n = 6) of plant material and five (n =5) for green tea, at a concentration level of 0.02% (w/v).

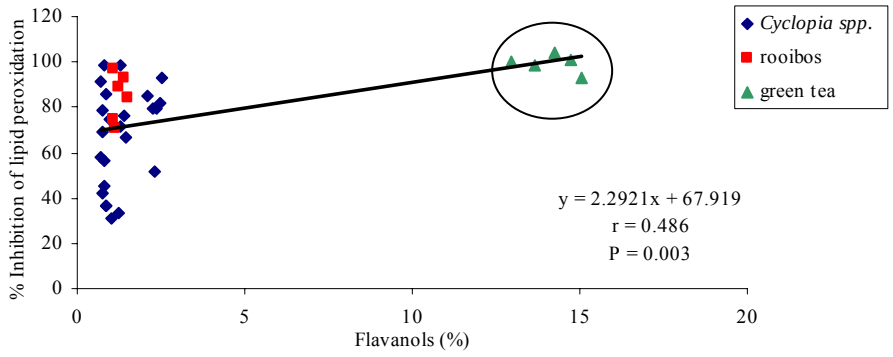
[b] The inhibition of lipid peroxidation in rat liver S9 by unfermented teas (0.0001% w/v) measured as the inhibition of MDA formation over 4 hrs at 37ºC. Values used for correlation were the average percentage inhibition calculated for unfermented *Cyclopia spp.* and rooibos tea, from six different batches (n = 6) of plant material and from five different batches (n = 5) for green tea

[c] Pearson correlation coefficient followed by the probability in brackets.

a)



b)



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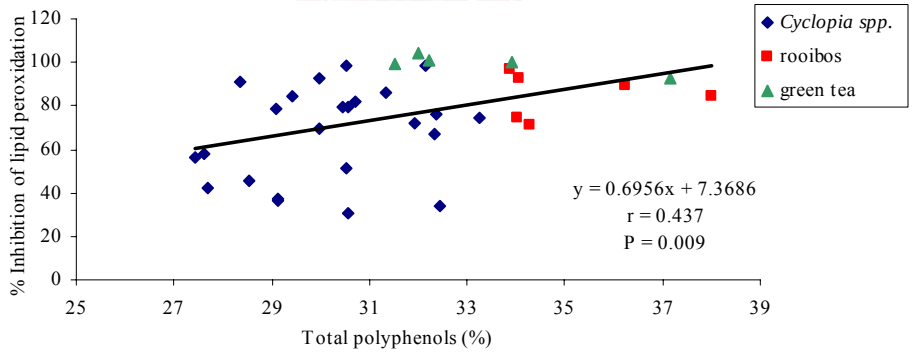


Figure 10 Correlation of the overall % $\Delta A_{450–425}$ in rat liver S9 in the presence of unfermented teas with a) their flavanol content, and their protection against lipid peroxidation with b) their flavanol and c) total polyphenol content.

Table 6 Separate correlation of stabilising[a] of cytochrome P450 and protection against lipid peroxidation[b] in rat liver S9 by unfermented *Cyclopia spp.*, rooibos and green teas with the levels of phenolic groups in the aqueous extracts.

Assay	Tea[c]	Total Polyphenols (g gallic acid equivalents 100 g^{-1})	Flavanols (g catechin equivalents 100 g^{-1})	Flavonols/Flavones (g quercetin equivalents 100 g^{-1})
Stability (% change in $\Delta A_{450-425}$)	*Cyclopia spp.* [d]	r = - 0.102[e] (P = 0.635)	r = 0.070 (P = 0.745)	r = - 0.031 (P = 0.886)
	Rooibos tea	r = 0.571 (P = 0.236)	r = 0.485 (P = 0.329)	r = 0.034 (P = 0.948)
	Green tea	r = 0.738 (P = 0.154)	**r = 0.824 (P = 0.086)**	r = - 0.340 (P = 0.575)
% Inhibition of lipid peroxidation	*Cyclopia spp.*	r = 0.253 (P = 0.232)	r = 0.305 (P = 0.148)	**r = -0.456 (P = 0.026)**
	Rooibos tea	r = 0.026 (P = 0.961)	r = 0.282 (P = 0.589)	r = 0.438 (P = 0.385)
	Green tea	**r = - 0.837 (P = 0.077)**	r = - 0.368 (P = 0.542)	r = 0.421 (P = 0.481)

[a] Stabilising ability of aqueous extracts (0.02% w/v) measured as the % change in $\Delta A_{450-425}$ in rat liver S9 that occur in the presence of tea extracts over 4 hrs at 37ºC.

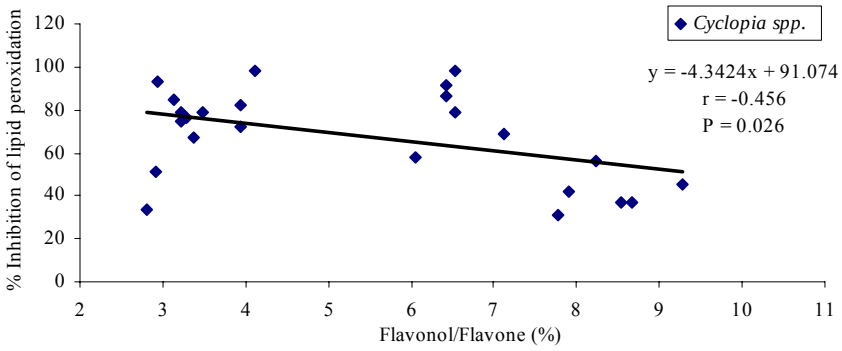
[b] The inhibition of lipid peroxidation in rat liver S9 by unfermented teas (0.0001% w/v) measured as the inhibition of MDA formation over 4 hrs at 37ºC. Values used for correlation were the average percentage inhibition calculated for unfermented *Cyclopia spp.*, rooibos and green tea.

[c] Six different samples of unfermented *Cyclopia spp.* and rooibos tea were obtained from different batches (n = 6) of plant material and from five (n = 5) different batches for green tea.

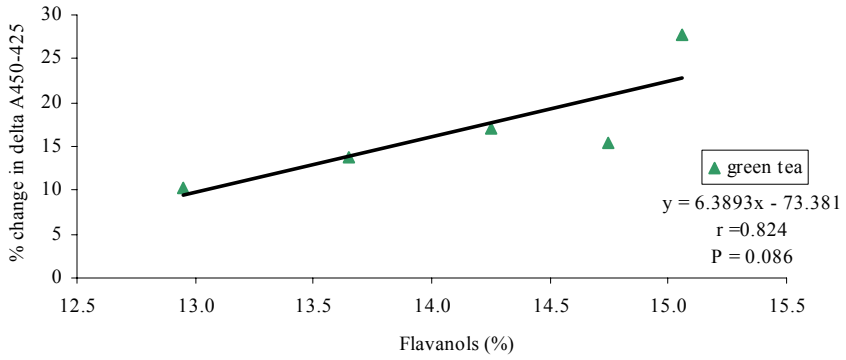
[d] The correlation of *Cyclopia spp.* phenolic composition was determined for unfermented *C. intermedia*, *C. subternata*, *C. genistoides* and *C. sessiliflora.*

[e] Pearson correlation coefficient followed by the probability in brackets.

a)



b)



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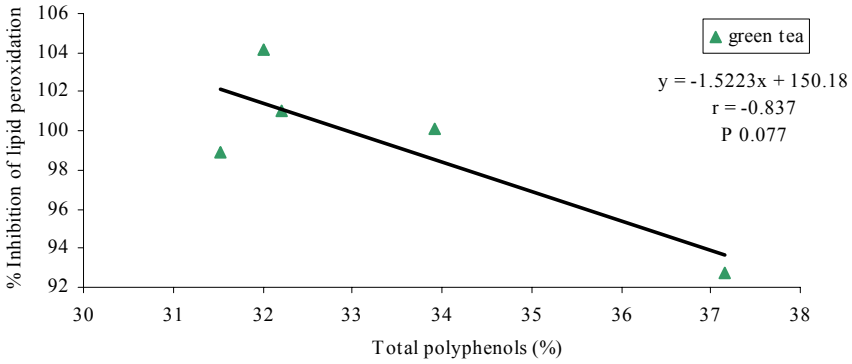


Figure 11 Separate correlations of a) the inhibition of lipid peroxidation in rat liver S9 in the presence of unfermented *Cyclopia spp.* with their flavonol/flavone content; b) the % change $\Delta A_{450–425}$ in S9 in the presence of green tea with the flavanol content and c) the inhibition of lipid peroxidation by green tea with the total polyphenol content

Table 7 Correlation of stabilising[a] of cytochrome P450 and protection against lipid peroxidation[b] in rat liver S9 with the levels of phenolic compounds in the aqueous extracts from unfermented *Cyclopia spp.*, rooibos and green teas

Tea[c]	Compound	Stabilising (% change in $\Delta A_{450-425}$)	% Inhibition of lipid peroxidation
Rooibos	Aspalathin	r = - 0.154[c] (P = 0.771)	r = - 0.125 (P = 0.813)
	Orientin	r = - 0.443 (P = 0.378)	r = 0.402 (P = 0.429)
	Iso-orientin	r = - 0.344 (P = 0.505)	r = 0.520 (P = 0.290)
Cyclopia spp.	Mangiferin	r = - 0.054 (P = 0.802)	**r = - 0.579 (P = 0.003)**
	Hesperidin	r = 0.162 (P = 0.448)	r = - 0.249 (P = 0.240)

[a] Stability of cytochrome P450 in rat liver S9 measured as the % change in $\Delta A_{450-425}$ in the presence of six different samples (n = 6) of unfermented *Cyclopia spp.* and unfermented rooibos at a concentration level of 0.02% (w/v).

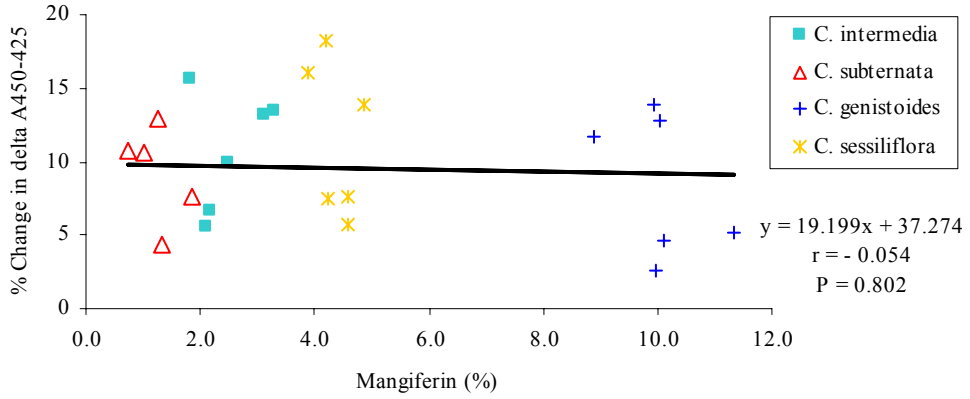
[b] The inhibition of lipid peroxidation in rat liver S9 by unfermented teas (0.0001% w/v) measured as the inhibition of MDA formation over 4 hrs at 37ºC. Values used for correlation were the average percentage inhibition calculated for unfermented *Cyclopia spp.* and rooibos from six different samples (n = 6).

[c] Aspalathin, orientin and iso-orientin content of fermented and unfermented rooibos tea determined with HPLC on a reversed phase LiChrospher 100 RP–18 C_{18} column with 2% formic acid in water and 100% methanol as eluents, expressed as a percentage of the aqueous extracts.

[d] Pearson correlation coefficient of the correlation of activity in different assays followed by the probability in brackets.

[e] Mangiferin and hesperidin content of fermented and unfermented *Cyclopia spp.* determined with reversed-phase HPLC analysis carried out on a Synergy MAX-RP C12 column with 100% acetonitrile and 2% acetic acid in water as eluents, expressed as a percentage of the aqueous extracts.

a)



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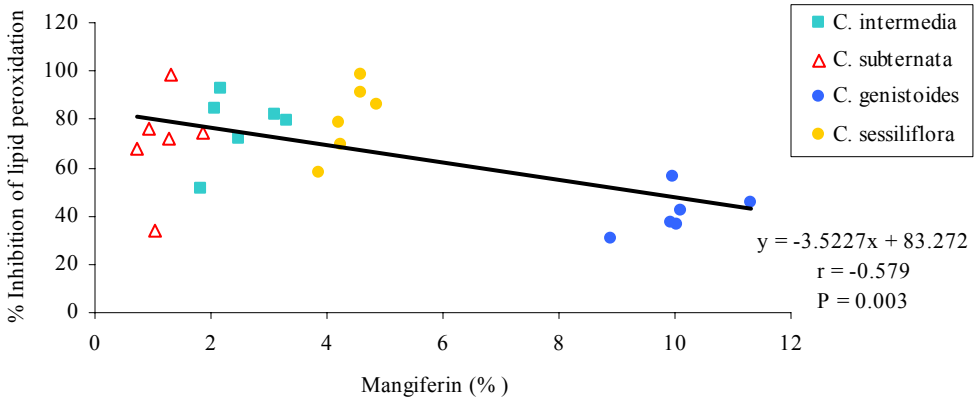

Figure 12 Correlation of a) the % change in $\Delta A_{450-425}$ and b) the inhibition of lipid peroxidation with the levels of mangiferin in unfermented *Cyclopia spp.*

Discussion

Cytochrome P450 is an important enzyme system responsible for the metabolism of procarcinogens resulting in the formation of highly reactive metabolites, with the ability to react with proteins and bind to DNA (Katiyar & Mukhtar, 1997; Omura, 1999). The most selective affect by which dietary components exert their effects on drug metabolism appears to be through interference with cytochrome P450 dependent metabolism (Yang *et al*., 1992), which has been reported as the important mechanism in the antimutagenic activity of green tea (Bu-Abbas *et al*., 1994; Bu-Abbas *et al*., 1997). Wang *et al*. (1987) indicated that carcinogen activation by cytochrome P450 would be modulated by binding with catechins from green tea. This assumption was based on the inhibition of cytochrome P450 dependent monooxygenase, NADPH-cytochrome *c* reductase and epoxide hydrolase enzyme activities in rat liver microsomes by green tea polyphenols, as well as the spectral changes in the difference spectra of microsomes from phenobarbital (PB) treated rats upon the addition of epicatechin derivatives from green tea (Wang *et al*., 1987). It was stated that alterations to the structure and function of cytochrome P450 achieved through the direct interaction of tea constituents with the enzyme may lead to altered rates and differential pathways of metabolism of mutagens and carcinogens (Wang *et al*., 1987).

Inclusion of an exogenous mammalian drug metabolising enzyme system in the *Salmonella* mutagenicity assay is necessitated by the inability of bacteria to metabolise chemicals via cytochrome P450, as in mammals and other vertebrates (Mortelmans & Zeiger, 2000). Studies on this mammalian activation system utilised in the *Salmonella* mutagenicity assay could provide important information regarding the mechanisms involved in the modulation of carcinogen metabolism *in vitro*.

The present study provided the first data on the *in vitro* interaction of rat liver cytochrome P450 with *Cyclopia spp.* and rooibos tea, as well as the first comparison to that of *Camellia sinensis.* This study also provided possible mechanisms involved in *in vitro* antimutagenic properties of these teas. Two important aspects investigated in the present study that could play a role in the extent of carcinogen metabolism *in vitro* were the stabilising of cytochrome P450 and the modulation of cytochrome P450 substrate binding by different teas and selected phenolic compounds.

The binding of carbon monoxide to dithionite-reduced microsomal hemoproteins results in a characteristic peak at 450 nm, while the appearance of an absorption peak at 420 nm has been suggested to be due to a denatured product of cytochrome P450 (Omura & Sato, 1964b).

Cytochrome P420 has been suggested to be a denatured product of cytochrome P450 due to its CO binding capacity and autoxidisability (Omura & Sato, 1964b). In the present study the difference in absorbance at 450 nm and 425 nm ($\Delta A_{450–425}$) was taken as an indication of cytochrome P450 stability in the liver homogenate fractions. It was found that calculation of the cytochrome P450 concentration varies between assays, while the $\Delta A_{450–425}$ value, including both the cytochrome P450 absorption maximum and the absorption maximum of the denatured product at 425 nm, was more sensitive and a more specific marker for cytochrome P450 stability. The decrease in the absorption at 450 nm accompanied with increased absorption at 425 nm in the present study, and not at 420 nm as reported (Omura & Sato, 1964b, Wang *et al*., 1987), could be the result of using Aroclor 1254 as opposed to PB.

The % change in $\Delta A_{450–425}$ over 4 hrs at 37ºC in the presence of tea indicated that the different teas effectively ($P < 0.05$) prevented the explicit decrease in cytochrome P450 stability observed in the rat liver S9 fraction in the absence of tea. A weak correlation ($r = 0.411$, $P = 0.013$) existed for the % change in $\Delta A_{450–425}$ in rat liver S9 in the presence of the unfermented teas with their antimutagenic activity, indicating that a higher percentage (less stability of cytochrome P450) is associated with higher antimutagenic activity against AFB_1. Less stability of cytochrome P450 imply that the enzyme would be active for a shorter period during the 48 h incubation step of the *Salmonella* mutagenicity assay than in the presence of tea. A prolonged period of enzyme activity as a result of the presence of tea or phenolic compounds with the ability to stabilise cytochrome P450 could possibly lead to higher concentrations of the active mutagenic metabolite. This in turn may result in an increased amount of revertant colonies in the *Salmonella* mutagenicity assay. The outcome therefore suggests that a strong stabiliser would have less antimutagenic potency, while a weak stabiliser would exhibit higher antimutagenic potency in the *Salmonella* mutagenicity assay. It would therefore be expected that rooibos tea, exhibiting a better stabilising effect than green tea, would demonstrate a weaker antimutagenic response. Findings obtained from the present investigation supported this hypothesis, as aqueous extracts from unfermented rooibos tea exhibited weaker antimutagenic activity against AFB_1 in the *Salmonella* mutagenicitiy assay, using tester strain TA100, when compared to green tea. The stabilising potential of unfermented *C. intermedia* (10.76% change in $\Delta A_{450–425}$) and unfermented *C. sessiliflora* (11.49% change in $\Delta A_{450–425}$) was markedly less than that of unfermented rooibos (3.30% change in $\Delta A_{450–425}$). As found for green tea, aqueous extracts from these unfermented species had significantly higher antimutagenic activity than unfermented rooibos tea against AFB_1.

Correlation of the levels of phenolic groups and the levels of individual compounds in the teas with their stabilising effect were performed to investigate the role of composition on the stability of cytochrome P450. No relevant correlations were observed with the levels of phenolic groups in the unfermented teas, but the flavanol content of green tea seemed to have a marginal ($P <= 0.824$, $P = 0.086$) effect on the % change in $\Delta A_{450-425}$. This implies that a higher flavanol content in green tea may be responsible for less stability of cytochrome P450.

The phenolic compounds, except hesperidin, demonstrated significant ($P < 0.05$) stabilising of cytochrome P450. EGCG demonstrated the highest stabilising effect on cytochrome P450, followed by luteolin, chrysoeriol and eriodictyol. The weaker stabilising effect demonstrated by aspalathin and mangiferin, and no stabilising by hesperidin, may be attributed to the presence of large sugar moieties, preventing interaction with cytochrome P450.

The mechanisms responsible for reduced stability of cytochrome P450 in the rat liver S9 fraction are not known at present. However, the instability of cytochrome P450 under aerobic conditions has been attributed to aerobic peroxidation of the microsomal phospholipids, due to the tight association of the enzyme with the microsomal architecture (Omura & Sato, 1964a). It was postulated that lipid peroxidation initiated by reactive oxygen species could affect membranal lipid peroxidation, consequently resulting in disrupted membrane integrity, leading to altered cytochrome P450 activity (Schaefer *et al.*, 1985).

The considerable decrease in cytochrome P450 stability (*ca.* 78% change in $\Delta A_{450-425}$) in the rat liver S9 fraction, was associated with the extensive increase in lipid peroxidation (1.760 nM MDA mg^{-1} protein) occurring in rat liver S9 under the same conditions used to assess stability of the enzyme. This led to investigation of the protection of unfermented teas against lipid peroxidation in S9, to asess whether antioxidant activity of the teas, in terms of the inhibition of lipid peroxidation, is related to their stabilising of cytochrome P450.

Although all the teas exhibited a high degree ($P < 0.05$) of protection against lipid peroxidation and significant ($P < 0.05$) stabilising of cytochrome P450, no correlation ($r = 0.018$, $P = 0.915$) between the stability and inhibition of lipid peroxidation was evident in the present investigation. This was attributed to different levels of activity in the respective assays, with green tea exhibiting the strongest inhibition of lipid peroxidation, but demonstrating the least stabilising of cytochrome P450. This could be related to green tea constituents, especially flavanols, that may exhibit prooxidant activity at high concentrations, resulting in enhanced lipid peroxidation. Prooxidant activity of green tea extracts (Yen *et al.*, 1997; Wanasundra & Shahidi, 1998) and green tea polyphenols (Roedig-Panman & Gordon, 1997) has been demonstrated.

Two abundant green tea flavanols, EGCG and (-) epicatechin (EC) (Balentine *et al*., 1997) have been shown to exhibit prooxidant properties such as generation of superoxide anion and the hydroxyl radicals (Azam *et al*., 2004). In the present study a relatively high concentration of aqueous extract (0.179 mg mL^{-1}) was used, compared to the relatively low concentration (0.001 mg mL^{-1}) used to assess inhibition of lipid peroxidation. At the low concentration of green tea used to assess protection against lipid peroxidation, prooxidant activity is likely to not play a role. Activity of unfermented rooibos was similar to that of unfermented *Cyclopia spp.* in terms of stabilising of cytochrome P450 and inhibition of lipid peroxidation, except for *C. genistoides* demonstrating significantly ($P < 0.05$) less protection against lipid peroxidation in rat liver S9. It appears that the relative concentrations of the individual flavonoids in the different teas could influence the cytochrome P450 stability by either exhibiting antioxidant and/or prooxidant effects. In this regard the major catechin (flavanol) in green tea, EGCG (Balentine *et al*., 1997) demonstrated better stabilising of cytochrome P450 than the major compound in unfermented rooibos (aspalathin) and unfermented *Cyclopia spp.* (mangiferin and hesperidin).

The relative stability of cytochrome P450 in microsomal preparations (very little decrease in stability detected) when compared to the S9 fraction, suggested that cytosolic factors are involved in mediating lipid peroxidation of the S9 fraction. Other factors that may play a role in lipid peroxidation in S9 observed in the present study, include the presence of iron in the liver homogenate fraction. Determining the extent of lipid peroxidation in microsomal preparations is suggested to provide further insight into the role of lipid peroxidation in cytochrome P450 stability. Additionally, the release of proteolitic enzymes during the preparation of the S9 fraction could be involved in the destruction of the protein constituent of membranes during the incubation of 48 hrs at 37ºC in the *Salmonella* mutagenicity assay (Dr. WCA Gelderblom, MRC, personal communications, 2004), resulting in decreased cytochrome P450 stability.

The weak correlation ($r = 0.475$, $P < 0.003$) observed for antimutagenic activity of teas, with their inhibition of lipid peroxidation, suggests that inhibition of lipid peroxidation may be of importance in protection against AFB_1-induced mutagenesis. This is demonstrated by green tea exhibiting the highest protection against lipid peroxidation and AFB_1-induced mutagenesis. Protection of lipid peroxidation by unfermented rooibos was similar ($P > 0.05$) to that of unfermented *Cyclopia spp.* except *C. genistoides,* with the same trend observed for antimutagenic activity. The mechanistic implications of this finding is not known at present.

The inhibition of lipid peroxidation was correlated with the levels of phenolic groups and levels of individual compounds in unfermented rooibos and *Cyclopia spp.*, to provide possible

explanations for the observed differences in the protective effect against lipid peroxidation. The total polyphenol content of the teas correlated weakly ($r = 0.437$, $P = 0.009$) with the observed inhibition of lipid peroxidation. Correlations of the activity of teas separately with the total polyphenol content, however, did not reveal any relevant correlations, except for a weak negative correlation ($r = -0.456$, $P = 0.026$) with the flavonol/flavone content of unfermented *Cyclopia spp*. Richards (2002) on the other hand reported a strong correlation ($r = 0.88$, $P < 0.0001$) for the total polyphenol content of fermented and unfermented *Cyclopia spp.* with the ability to inhibit Fe^{2+}-induced microsomal lipid peroxidation. This discrepancy may be the result of the differences in S9 and microsomal mixtures already discussed, as well as the addition of Fe^{2+} (Richards, 2002) to the reaction mixture.

Another determining factor that may influence the mutagenic outcome in the *Salmonella* mutagenicity assay is the inhibition of carcinogen binding and the subsequent activation by binding of tea constituents to cytochrome P450. In the present study this was investigated by monitoring the modulating effect of aqueous extracts from unfermented teas and selected phenolic compounds on the aniline-induced Type II binding to cytochrome P450 in rat liver microsomes obtained from Aroclor 1254 treated rats. Type II binding is associated with heme ligation of cytochrome P450 (Schenkman *et al.* 1981) and thus indicative of interaction with the heme iron of cytochrome P450 (Schenkman *et al*., 1972). The modulation of this binding type by tea constituents may thus be an indication of the type of interaction exerted by tea with cytochrome P450. No information of previous investigations of this nature could be found in the literature. In a somewhat related study, Wang *et al*. (1987), however, demonstrated that addition of green tea epicatechin derivatives to hepatic microsomes from PB-treated rats result in spectral changes characteristic of the reverse Type I in difference spectra.

Spectophotometrically observable transitions in hemoproteins are attributed to various changes in the electronic configuration of their iron porphyrin prosthetic group (Schenkman *et al*., 1981). These changes are evident for hemoproteins upon binding of substrates, acceptance or transfer of an electron and/or binding or displacement of a ligand to or from the heme iron. The remarkable absorption changes that occur in the Soret region (the region of hemoprotein absorption around 400 nm) of the UV-visible spectrum is linked to substrate interaction or a redox change in the hemoproteins. Modifying effects to the cytochrome P450 protein as well as the effects of any bound substrate determine the position of the absorption peak in the spectrum and the magnitude of absorption (Schenkman *et al*., 1981). Spectral interaction of substrates of the microsomal mixed-function oxidase with cytochrome P450 has been defined in terms of

spectral dissociation constant (K_s) and maximal spectral interaction (ΔA_{max}) (Remmer *et al*, 1966; Schenkman *et al*., 1967). In the present study ΔA_{max} (calculated from the minimum and maximum absorbance) was used to compare the modulating effect of unfermented teas and selected phenolic compounds on the aniline-induced Type II binding spectra of rat liver microsomes. The Type II spectral change is characterized by the appearance of an absorption peak at approximately 430 nm and a minimum at 390 (Remmer *et* al., 1966; Schenkman *et al*., 1967).

Differences in the interaction of constituents of the different teas with cytochrome P450 were manifested by the dissimilar modulating effects of aqueous tea extracts. Unfermented *Cyclopia spp.* and rooibos resulted in reduced maximum and increased minimum absorbance to a varying degree. Unfermented rooibos and *C. sessiliflora* demonstrated similar ($P > 0.05$) modulating effects to *C. subternata* and *C. genistoides*, but a significantly higher degree of modulation than *C. intermedia.* The modulating effect of green tea demonstrated in the difference spectra was opposite, in that it resulted in an increased absorption at the maximum and decreased absorption at the minimum. This implies that the binding of aniline to cytochrome P450 is stimulated, or that a compound(s) in green tea also exerts type II binding. This effect can, however, not be ascribed to the modulation by EGCG, the major catechin present in green tea, since EGCG had an opposite effect on the aniline-induced binding spectra of microsomal cytochrome P450. EGCG is also known to exhibit a Reverse Type I binding with microsomal cytochrome P450 (Wang *et al*., 1987).

Luteolin, hesperetin, chrysoeriol and eriodictyol exhibited a high percentage of modulation of Type II binding. The shift in the minimum absorbance of the aniline-induced Type II difference spectra in rat liver microsomes observed in the presence of luteolin, hesperetin, chrysoeriol and eriodictyol was not observed with the glycosides aspalathin, mangiferin and hesperidin or the catechin EGCG. This shift implies that the compounds interact with cytochrome P450 and may also exhibit different binding types, as has been demonstrated for EGCG (Wang *et al*., 1987). The Reverse Type I binding that has been reported for EGCG differs from the Type II binding in terms of the minimum and maximum absorbance. This type of spectrum is thought to be the result of displacement of the distal ligand (water molecule or hydroxide ion) combined with substrate binding to the hydrophobic region of the heme pocket, instead of heme ligation (Type II) (Schenkman *et al*.,1981).

The weak modulation of cytochrome P450 substrate binding to aniline exerted by the glycosides, aspalathin, mangiferin and hesperidin, can possibly be explained by the presence of

large polar substitutes (sugar moieties). Although the aniline-induced Type II substrate binding to cytochrome P450 is modulated by the tea constituents and phenolic compounds, it is not known whether simultaneous binding to the same site of cytchrome P450 may play a role in the observed modulations. The contributions of two compounds that bind to the same site of cytochrome P450, thus eliciting the same type of spectral change, cannot be separated with spectrophotometric determinations (Van den Berg *et al*., 1979). The simultaneous binding of two compounds to the same site on cytochrome P450 may be examined by measuring the spectral changes elicited by several concentrations of one substrate in the presence of varying concentration of the other (Van den Berg *et al*, 1979). A study of this kind is recommended for future research to provide insight into the modulations observed here. The first compound (S) is added to the sample cuvette, whereas the second (I) is present in both sample and reference cuvettes (Van den Berg *et al*, 1979). In this study, tea extracts/phenolic compounds (I) interacted with cytochrome P450 before the addition of aniline (S) and the A_{max} values were compared to that of the control (absence of tea or phenolic compounds), to determine if the tea or compounds modulated the binding of aniline to cytochrome P450. It is however important to note that the difference spectra recorded is not the spectral change elucidated by tea/phenolic compounds, but the spectral changes produced by both tea/phenolic compound and aniline in the sample cuvette, minus the spectrum of the tea/phenolic compound alone in the reference cell (Van den Berg *et al*., 1979).

The spectral changes induced by the selected phenolic compounds are not known, except for EGCG that has been shown to induces a Reverse Type I spectral change in microsomes from PB treated rats (Wang *et al*., 1987). The spectral change induced by tea extracts cannot be predicted, since it contains such a complex mixture of phenolic compounds, differing immensely for *Cyclopia spp.* (Ferreira *et al*., 1998; Kamara *et al*., 2003; Kamara *et al*., 2004), rooibos (Rabe *et al*., 1994; Ferreira *et al*., 1995; Marais *et al*., 1998; Marais *et al*., 2000; Bramati *et al*., 2002) and green tea (Balentine *et al*., 1997; Chu, 1997; Wiseman *et al.*, 1997; Beecher *et al.,* 1999). Investigation into the binding types of the major phenolic compounds in the respective teas is recommended to determine the type of interaction with cytochrome P450. The type of interaction elicited by the mutagen may also provide valuable information and indicate whether the compounds may compete with the mutagens for binding to cytochrome P450.

The modulating effects on the Type II binding of aniline to cytochrome P450, by the different tea constituents, observed in the present study cannot necessarily be attributed to competitive inhibition, since subtraction of the spectral change induced by one of the substrates,

by means of calculation or by recording difference spectroscopy, results in spectra which is too small. Only a decrease in ΔA_{max} in addition to an increase in K_s may indicate the competitive binding of two compounds exhibiting the same type of spectral interaction (Schenkman *et al*., 1972).

It can thus not be concluded from the results whether the observed modulations are a result of competition of tea constituents with aniline for the same binding site on cytochrome P450 or an effect of simultaneous binding to the same binding site. The observed modulations, however, indicate that tea constituents may affect the binding of type II substrates to cytochrome P450, and it can be postulated that these effects could be responsible for antimutagenic activity.

Conclusions

The results obtained in the present study indicate that the stabilising effects of tea extracts may play a role in antimutagenic potency against AFB_1 *in vitro.* Lipid peroxidation was associated with the decreased stability of cytochrome P450 in rat liver S9. Inhibition of lipid peroxidation and stabilising of cytochrome P450 in S9 by the teas were observed, but the order of activity for the teas differed in the respective assays, which may be a result of the concentration levels used. The stabilising effect and inhibition of lipid peroxidation could, however, not be fully explained by the phenolic composition of the respective teas. Modulation of binding of aniline to cytochrome P450 indicated that possible interaction between tea constituents and cytochrome P450 occur in rat liver microsomes. It was established that the different teas and selected phenolic compounds differentially modulate aniline-induced Type II substrated binding by cytochrome P450. This study presents a platform for future research on the interactions of tea constituents of *Cyclopia spp.* and rooibos tea and major phenolic compounds in the respective teas with cytochrome P450.

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Chapter 5

General Discussion and Conclusions

A modern lifestyle is closely associated with the exposure to several mutagenic and carcinogenic risk factors, e.g. cigarette smoke, infection and inflammation, nutrition and dietary factors and exposure to environmental chemicals (Sugimura, 2000), of which avoidance appears to be troublesome, even impossible (De Flora, 1998), resulting in a need to counteract their genotoxic effects by dietary antimutagens and anticarcinogens regularly and continuously consumed. Identification of chemopreventive compounds in natural dietary elements such as plants or beverages is of particular importance in that it provides means of cancer prevention through commonly consumed compounds (Wattenberg, 1996). In this regard moderate tea (*Camellia sinensis*) consumption (5 cups/day or an extract of about 11 g of tea) was suggested to reduce the risk of certain types of human cancer (Apostolides *et al*., 1996).

Consumer concern regarding the detrimental effects of caffeine on health has increased consumption of decaffeinated teas, and the revival in consumption of "natural foods" has lead to herbal teas often being consumed as alternative to other beverages (Stavric *et al*., 1996). Dramatic growth in the market for the indigenous South African herbal teas, rooibos (Snyman, 2000) and honeybush teas (De Villiers, 2004) has been experienced, mainly reflecting increased popularity due to apparent health effects and the development of the international market (Dr. E Joubert, ARC Infruitec-Neitvoorbij, Stellenbosch, personal communications, 2004). Herbal teas are perceived to have many beneficial health effects, but possible risk associated with their use is not clearly understood and needs clarification (Stavric *et al*., 1996).

Green tea (*Camellia sinensis*) has well recognised antimutagenic activity against several mutagens (Kada *et al*., 1985; Mukhtar *et al*., 1992; Weisburger *et al*., 1996; Chen & Yen, 1997; Kuroda & Hara, 1999; Siess *et al*., 2000). Antimutagenic activity of rooibos tea and *C. intermedia* (honeybush tea) has been demonstrated against two indirect acting mutagens, i.e. 2-acetylaminofluorene (2-AAF) and aflatoxin B_1 (AFB_1), and to a lesser extent against three direct acting mutagens, methyl methanesulfonate (MMS), cumolhydroperoxide (CHP) and hydrogen peroxide (H_2O_2) (Marnewick *et al*., 2000). In the first comparative study on the antimutagenic activity of economic important *Cyclopia spp.*, including *C. intermedia*, Richards (2002) established that fermented and unfermented *C. intermedia*, *C. subternata* and *C. sessiliflora* protect to varying degrees against metabolically activated 2-AAF in the *Salmonella* mutagenicity

assay using tester strain TA98. Fermented *C. genistoides* also exhibited antimutagenic activity against 2-AAF-induced mutagenesis, but its unfermented counterpart enhanced mutagenicity.

The present study served as a follow-up to the investigation of the antimutagenic potential of commercially important *Cyclopia spp.*, started by Richards (2002), and the same aqueous extracts were subjected to testing. Antimutagenic activity was determined in terms of the ability to inhibit metabolically activated AFB_1 in the *Salmonella* mutagenicity assay, using tester strain TA100, and rooibos and *Camellia sinensis* i.e. black, oolong and green teas were included to serve as benchmarks. Fermented and unfermented *Cyclopia spp.* demonstrated less antimutagenic activity than black and oolong teas, while *Cyclopia spp.* except *C. genistoides*, exhibited similar protection than fermented rooibos and green teas. The extent of antimutagenic activity of fermented and unfermented *Cyclopia spp.* against AFB_1, as well as their relative efficacy differed when compared to their activity against 2-AAF (Richards, 2002), at the same concentration level (Table 1) as the antimutagenic activity of *Cyclopia spp.* was higher against AFB_1 than that reported against 2-AAF (Richards, 2002). Unfermented *C. genistoides* enhanced the mutagenicity of 2-AAF (Richards, 2002), but it was established in the present study that, although less effective than other unfermented *Cyclopia spp.*, unfermented *C. genistoides* protects against AFB_1-induced mutagenesis. Unfermented *C. intermedia, C. sessiliflora* and *C. substernata* demonstrated similar inhibition of AFB_1 and 2-AAF-induced mutagenesis (Richards, 2002). Of the fermented teas, *C. sessiliflora* and *C. subternata* demonstrated the highest inhibition of 2-AAF-induced mutagenesis (Richards, 2002), but *C. genistoides* and *C. sessiliflora* demonstrated the highest antimutagenic activity against AFB_1. Differences in the protection against the indirect acting mutagens 2-AAF and AFB_1 by *C. intermedia* have also been reported (Marnewick *et al*., 2000). The results of these studies clearly indicate that the extent of antimutagenic activity may vary with the mutagen used. The relative differences in the antimutagenic potency of *Cyclopia spp.* against 2-AAF and AFB_1 were suggested to be related to different pathways of metabolic activation of the carcinogens that could be differentially affected by the different teas (Marnewick *et al*., 2000).

In comparative studies regarding the antimutagenic activity of different teas in the *Salmonella* mutagenicity assay, several aspects need to be considered. The different effects of tea extracts on cytochrome P450, contained in the exogenous mammalian metabolic activation system included in the *Salmonella* mutagenicity assay, may cause results not to be an accurate reflection of antimutagenic potential. Examination of interaction of the aqueous extracts with Aroclor 1254-induced rat hepatic cytochrome P450 in terms of stabilising the enzyme in rat liver

S9 and modulation of aniline-induced Type II substrate binding in the microsomal fraction, were included to provide possible insight into the mechanisms involved in the antimutagenic activity of the respective teas. In the event of tea or phenolic compounds resulting in a prolonged period of enzyme (cytochrome P450) activity as a result of the stabilising effect, it may lead to higher concentrations of the active mutagenic metabolite in the *Salmonella* mutagenicity assay. It is therefore suggested that a strong stabiliser of cytochrome P450 would have less antimutagenic potency, while a weak stabiliser would exhibit higher antimutagenic potency in the *Salmonella* mutagenicity assay. Differences in the antimutagenic activity of the unfermented teas, i.e. unfermented *Cyclopia spp.*, unfermented rooibos and green tea, were associated with differential stabilising of cytochrome P450 by the teas. In this regard unfermented rooibos tea exhibited less antimutagenic activity against AFB_1 than green tea, which could be related to the increased stabilising of cytochrome P450 by unfermented rooibos. In the event of tea enhancing the mutagenicity, but not being a mutagen on its own, as in the case of unfermented *C. genistoides* against 2-AAF (Richards, 2002), it may be due to the stabilising effect on cytochrome P450 being more prominent than the factors responsible for antimutagenic activity. It appears that a balance exist between the modifying factors influencing mutagenicity and the inhibitory factors responsible for antimutagenic activity, such as the inhibition of metabolic activation of a mutagen through the modulation of cytochrome P450 substrate binding.

Decreased stability of cytochrome P450 was associated with an increase in lipid peroxidation in the S9 fraction. This was expected since Schaefer *et al*. (1985) postulated that cytochrome P450 can be destroyed by lipid peroxidation initiated by reactive oxygen species. This postulation was based on the facts that oxidation of fatty acids of liver microsomal membranes results in destruction of the membrane architecture (Vatsis *et al*., 1974) and cytochrome P450 is tightly associated with the microsomal membrane (Omura & Sato, 1964). Aqueous extracts from the respective unfermented teas effectively counteracted the decrease in stability of cytochrome P450 and provided significant protection against lipid peroxidation observed in S9. This suggested that the inhibition of lipid peroxidation by the teas could be related to its stabilising effects on cytochrome P450. No correlation ($r = 0.018$, $P = 0.915$) was, however, observed for these activities. This was attributed to different mechanisms in the respective assays and/or probable prooxidant activity of green tea at the high concentration level of the unfermented teas in the stability studies as opposed to the strong antioxidant activity exhibited by green tea (at a much lower concentration) in the inhibition of lipid peroxidation**.** However, the protection by aqueous extracts from unfermented teas against AFB_1 appeared to be

related to their protection against lipid peroxidation in S9. Green tea offered the highest protection, while unfermented *C. genistoides* demonstrated the least activity in both assays. This paradox needs to be investigated in future research.

Compositional analysis of the teas was aimed at providing possible insight into phenolic groups and compounds of importance in the biological activities, i.e. antimutagenicity, stabilising of cytochrome P450 and inhibition of lipid peroxidation, exerted by the teas. The total polyphenol, flavanol and flavonol/flavone contents did not appear to be related to the antimutagenic activity of *Camellia sinensis* teas or the inhibition of lipid peroxidation by green tea. The flavanol content of green tea, however, appeared to marginally ($P < 0.1$) affect the stabilising effect of green tea on cytochrome P450. This implied that the decreased stability of cytochrome P450 is associated with an increase in the flavanol content, which could be related to prooxidant activity of flavanols at high concentrations, as suggested above. Several studies have demonstrated prooxidant activity for green tea flavanols (Yen *et al*., 1997; Wanasundra & Shahidi, 1998), most notably for EGCG (Azam *et al*., 2004), the most abundant flavanol in green tea (Balentine *et al*, 1997).

The higher flavonol/flavone content of fermented rooibos tea compared to unfermented rooibos, was associated with its higher antimutagenic activity against AFB_1. Increased levels of the flavones, orientin and iso-orientin, were associated with increased antimutagenic activity of fermented and unfermented rooibos tea. In contrast, both orientin and iso-orientin have been shown to have poor antimutagenic activity against metabolically activated AFB_1 and 2-AAF (Snijman *et al*., 2004). An increase in the content of the major compound in unfermented rooibos tea, aspalathin, appeared to be associated with decreased antimutagenic activity. The antimutagenic activity of aspalathin has, however, been reported to be similar to that of orientin and iso-orientin against AFB_1 at a concentration level of 0.04 mM and 0.8 mM (Snijman *et al*., 2004). Other compounds in rooibos tea may be responsible for the observed effects and it can be speculated that these contradictory results may be the effect of the levels of aspalathin, orientin and iso-orientin correlating with the activities investigated, by chance.

The levels of aspalathin, orientin and iso-orientin in the unfermented tea extracts, did not appear to play a role in the stabilising effect on cytochrome P450 or their inhibition of lipid peroxidation, as no correlations were evident. This was unexpected, since Snijman *et al*. (2004) demonstrated that the inhibition of Fe^{2+}-induced lipid peroxidation by these compounds differ substantially in rat liver microsomes. Aspalathin was more effective than orientin, while iso-orientin had very low efficacy (Snijman *et al*., 2004). Failure to demonstrate a correlation

between the aspalathin content of unfermented rooibos tea and its inhibitory effect of lipid peroxidation in the present study, could be attributed to possible oxidation and/or metabolism of aspalathin, during the 4 hr incubation period at 37ºC. The conversion of the dihydrochalcone, aspalathin, to the intermediate products, 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin, has been observed in an ethanolic solution (Koeppen & Roux, 1966). Important considerations are the comparison of the activity of flavonoids at different concentration levels in different mediums, and the possibility of prooxidant activity at high concentrations. Although Snijman *et al*. (2004) reported a high inhibition of Fe^{2+}-induced lipid peroxidation in rat liver microsomes, Winterton (1999) reported that an ethyl acetate soluble fraction of unfermented rooibos, containing high levels of aspalathin (> 50%) was not very effective in inhibition of linoleic acid peroxidation.

The total polyphenol and flavanol contents of *Cyclopia spp.* appeared to influence the antimutagenic activity observed against AFB_1, while the levels of the respective phenolic groups did not play a role in the stabilising of cytochrome P450 or inhibition of lipid peroxidation by unfermented *Cyclopia spp.* Investigation with larger sample sizes of the different species is recommended to demonstrate more accurate correlations. Inclusion of the fermented samples in studying the stabilising effect on cytochrome P450 and the protection against lipid peroxidation by *Cyclopia spp.* and rooibos tea may provide further insight into the apparent mechanisms involved in antimutagenic activity of these teas.

The activity of *C. genistoides* in the different assays did not appear to be related to the activities observed for the other *Cyclopia spp.* Fermented *C. genistoides* demonstrated markedly higher protection against AFB_1-induced mutagenesis than other fermented *Cyclopia spp.*, while its unfermented counterpart demonstrated the least antimutagenic activity and protection against lipid peroxidation of the unfermented species. Unfermented *C. genistoides*, however, exhibited similar stabilising ability to rooibos and *C. subternata*.. The effect of fermentation on the total polyphenol, flavanol and flavonol/flavone contents of *C. genistoides* appeared to be less than that for other *Cyclopia spp.* Future research on the effect of fermentation on *C. genistoides* is recommended to investigate the increase of antimutagenic activity evident after fermentation. The differences in activity could also be ascribed to the phenolic profile of *C. genistoides* differing from that of other *Cyclopia spp.* Narirutin is not present and mangiferin levels are much higher in unfermented *C. genistoides* than in the other *Cyclopia spp.* The mangiferin content of unfermented *Cyclopia spp.* did, however, appear to be of no importance in either the observed stabilising effect on cytochrome P450 and inhibition of lipid peroxidation in S9, or in

the antimutagenic activity of fermented and unfermented *Cyclopia spp.* This could be explained by the relatively weak antimutagenic activity exhibited by mangiferin against AFB_1. Richards (2002) demonstrated a weak, but significant correlation between mangiferin/isomangiferin content of the aqueous extracts and their ability to inhibit Fe^{2+}-induced rat liver microsomal lipid peroxidation. In this case, chelation of Fe^{2+} by mangiferin may be an important protective factor, explaining the difference in apparent effects.

Other differences in the phenolic profiles of *Cyclopia spp.* entailed the presence of hesperetin and eriocitrin in all the species investigated, except *C. subternata* and *C. intermedia*, respectively. As the differences in the phenolic profile of *Cyclopia spp.* could be responsible for their relative efficacy in the different assays, future research on the phenolic profiles of *Cyclopia spp.*, especially the presence of high molecular weight compounds such as proanthocyanidins and non-flavonoids, as well as the activities of individual compounds, may provide further understanding of the biological activities observed here. Many questions about the role of phenolic composition in the antimutagenic activity of teas remain to be answered and seem to be complicated by vast differences in phenolic profiles between different teas (*Camellia sinensis*, rooibos and *Cyclopia spp.*). Correlation of antimutagenic activity of tea extracts and composition thereof should be investigated with more samples in future, to clarify whether an effect such as the negative correlation of aspalathin with antimutagenic activity of rooibos tea was a result of sample selection. The role of phenolic composition in biological activity of the respective *Cyclopia spp.* should also be investigated separately in future, to obtain a clear understanding of important groups or compounds.

Unfermented rooibos, unfermented *Cyclopia spp.* and green tea demonstrated modulation of the binding of aniline to cytochrome P450. The modulating effect demonstrated of green tea, was opposite to that exhibited by the other teas, which was interpreted as an indication of different mechanisms involved in the interaction of green tea with cytochrome P450. Future research on the different interaction of different teas, or their phenolic constituents, with cytochrome P450 may provide further explanation into different levels of antimutagenic activity.

Investigating the activities of selected phenolic compounds in terms of antimutagenic acitivity against AFB_1, stabilising effects on cytochrome P450 and modulation of substrate binding in the different assays provided additional insight into their role. The glycosides aspalathin, hesperidin and mangiferin demonstrated the least activity of the phenolic compounds compared, which was attributed to the presence of large polar substituents, i.e. the sugar moiety. Edenharder & Tang (1997) reported that antimutagenic activity of glycosides are less than that of

their corresponding aglycones when using nitroarenes as mutagen in the *Salmonella* mutagenicity assay. This may be related to attachment of large polar substituents to flavonoids (Edenharder *et al*., 1993). The relatively weak to moderate activity of glycosides as antimutagens and stabilisers of cytochrome P450, may also be related to reduced capacity to penetrate into the membrane and so to affect membrane-dependent processes, which are of importance here, since cytochrome P450 is membrane bound (Omura & Sato, 1964). The large sugar moiety may also obstruct the interaction of compounds with cytochrome P450. Distinct structure-antimutagenicity relationships against several mutagens, including AFB_1, have been demonstrated for several flavonoids (Francis *et al*., 1989; Edenharder *et al*., 1993; Das *et al*., 1994; Edenharder & Tang, 1997). In contrast, structurally diverse flavonoids, e.g. the flavones, luteolin and chrysoeriol, the flavanones, hesperetin and eriodictyol, and the flavanol, EGCG, exhibited similar activity in terms of stabilising of cytochrome P450, protection against AFB_1-induced mutagenesis and the degree of modulation of cytochrome P450 substrate binding, except for the modulating effect of EGCG that was less. It is suggested that the importance of flavonoid structure in the antimutagenic activity against AFB_1 in tester strain TA100, in terms of differences in activity, was masked by the high inhibition values obtained at the concentration levels used. Future investigations at lower concentrations as well as dose response studies, may provide insight into the importance of structural differences of flavonoids in biological activities.

Another consideration is that i*n vitro* antimutagenic activity of flavonoids may vary when determined against different mutagens and different *Salmonella* strains (Edenharder *et al*., 1993). The antimutagenic activity of the major compounds in *Cyclopia spp.*, the xanthone mangiferin, and the flavanone, hesperidin, against AFB_1 differed from that against 2-AAF (Richards, 2002), when compared at the same concentration level (Table 2). Antimutagenic activity of mangiferin (66.35%) was considerably higher against AFB_1 compared to the weak protection (15.65%) against 2-AAF (Richards, 2002). Whilst hesperidin inhibited AFB_1-induced mutagenesis, it enhanced 2-AAF-induced mutagenesis. A similar trend was observed for another flavanone, eriodictyol. Luteolin demonstrated a high level of protection against both AFB_1 in the present study and 2-AAF (Richards, 2002).

Better stabilising of cytochrome P450 by the phenolic compounds was not associated with decreased antimutagenic activity against AFB_1 as demonstrated for the teas, implying that antimutagenic activity of the individual compounds differ from that of the tea and that synergistic mechanisms may be of importance in the tea extracts. EGCG demonstrated very high antimutagenic activity as well as the highest stabilising effect of the phenolic compounds on

Table 1 Antimutagenic activity of aqueous extracts of fermented and unfermented *Cyclopia spp.* against AFB_1 in the present study compared to antimutagenic activity against 2-AAF, as determined by Richards (2002), at a concentration level of 2 mg aqueous extract per plate.

	AFB_1[a] (20 ng per plate)	**2-AAF[ab] (5 µg per plate)**
C. intermedia Fermented	79.21	56.71
C. intermedia Unfermented	97.28	94.18
C. subternata Fermented	78.68	75.86
*C. subternat*a Unfermented	96.54	83.46
C. genistoides Fermented	87.87	48.84
C. genistoides Unfermented	83.24	-60.62
C. sessiliflora Fermented	85.61	81.39
C. sessiliflora Unfermented	98.58	93.89

[a] Values represent the mean percentage inhibition of six samples (n = 6) with five assay replications, except for antimutagenic activity of *C. genistoides* and *C. sessiliflora* against 2-AAF, where values represent the means of seven samples (n = 7).

[b] The values reported for the inhibition of 2-AAF-induced mutagenesis (Richards, 2002) were recalculated by subtracting the spontaneous revertant count for comparative purposes.

Table 2 Antimutagenic activity of phenolic compounds present in fermented and unfermented *Cyclopia spp.* against AFB_1 in the present study compared to antimutagenic activity against 2-AAF, as determined by Richards (2002), at a concentration level of 0.3 mM per plate.

	AFB_1[a] (20 ng per plate)	**2-AAF[ab] (5 µg per plate)**
Mangiferin	66.35	15.65
Hesperidin	69.44	-24.75
Luteolin	82.49	98.70
Eriodictyol	88.39	-53.58

[a] Values represent the mean percentage inhibition of five assay replications.

[b] The values reported for the inhibition of 2-AAF-induced mutagenesis (Richards, 2002) were recalculated by subtracting the spontaneous revertant count for comparative purposes.

Apart from EGCG, the modulating effect of the phenolic compounds on cytochrome P450 Type II substrate binding appeared to be related to their antimutagenic activity against AFB_1, thus implying that the level of interaction of these compounds with cytochrome P450 may be a determining factor in antimutagenic activity.

cytochrome P450 in rat liver S9. In contrast, it demonstrated very little modulation of binding, which was attributed to EGCG interacting with cytochrome P450 by binding at a different site, demonstrated by the reverse Type I binding upon addition of EGCG to rat liver microsomes (Wang *et al.*, 1987), than that responsible for Type II binding.

Antimutagenic potency of compounds at the same concentration level (molarity base) should, however, not be interpreted as a reflection of their possible contribution to the antimutagenic activity of the tea extracts, since actual levels in the tea differ and synergistic effects of compounds are not taken into account. It was demonstrated in the present study that the levels of these compounds in the *Salmonella* mutagenicity assay, administered as part of the tea extracts, are not nearly comparable to the concentrations used for comparison of the phenolic compounds individually. This would also be applicable to the investigation on the stabilising effect of cytochrome P450 and of the modulation of Type II substrate binding by the selected phenolic compounds.

An important consideration is that *in vivo* antimutagenic activity of flavonoids may be different from that determined *in vitro* due to intestinal absorption and bioavailability of flavonoids. Glycosides, for example, are metabolized to their aglycones by intestinal microflora before absorption through the colon barrier (Schalbert & Williamson, 2000; Walle, 2004). The inhibitory effect of flavonoids in the diet on AFB_1 initiated hepatocarcinogenesis in rats is influenced by the flavonoid structure (Siess *et al.*, 2000) in that only non-polar flavonoids without free hydroxyl groups showed a protective effect (Siess *et al.*, 2000). In contrast the phenolic compounds (all containing free hydroxyl groups) demonstrated a protective effect against AFB_1-induced mutagenesis in the present study. In this regard, liver cytosolic fractions from rats that had received fermented and unfermented rooibos and *C. intermedia* have been shown to protect against AFB_1-induced mutagenesis *ex vivo*, while green and black tea demonstrated no cytocolic protection (Marnewick *et al*., 2004).

In the present study protection against a mutagen, AFB_1, associated with liver cancer in human populations, by aqueous tea extracts, representative of the phenolic composition in a cup of tea, contributed to the results being relative for general human health. Although the results obtained in *in vitro* studies cannot necessarily be extrapolated to the effect on humans, it serves as

an important tool for investigating potential protective or adverse effects of natural dietary elements such as tea. In addition, it can be used to compare relative activity, and investigate the mechanisms involved in observed effects.

The present study demonstrated that results obtained with the *Salmonella* mutagenicity assay should not be interpreted in isolation and factors such as kinetics in terms of the stability of the activating enzyme should be taken into account. Identification of such factors could aid in interpretation of results obtained with *in vitro* tests being more applicable for prediction of activity *in vivo.*

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Addendum A

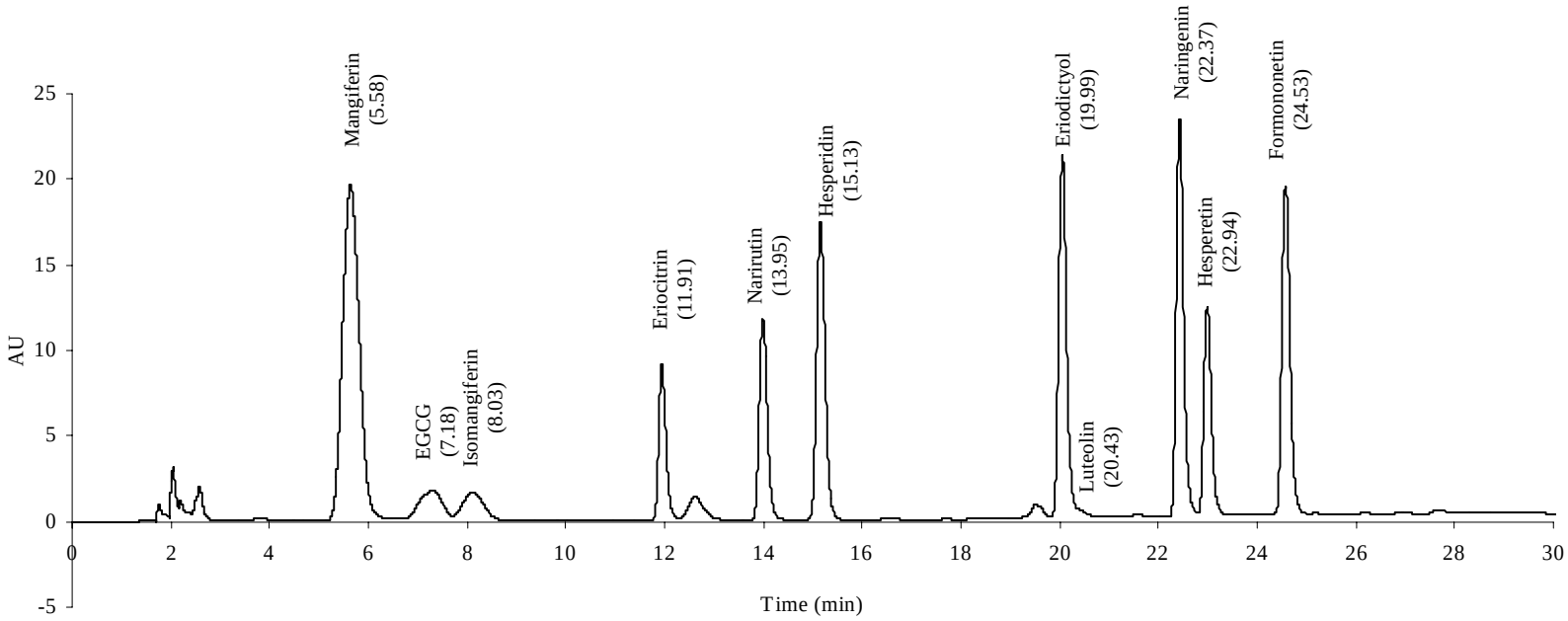


Figure 1 Chromatogram of the standard mixture of polyphenols used for the quantification of individual compounds in *Cyclopia spp.* The retention time of the compounds is given in brackets. Detection was at 280 nm.

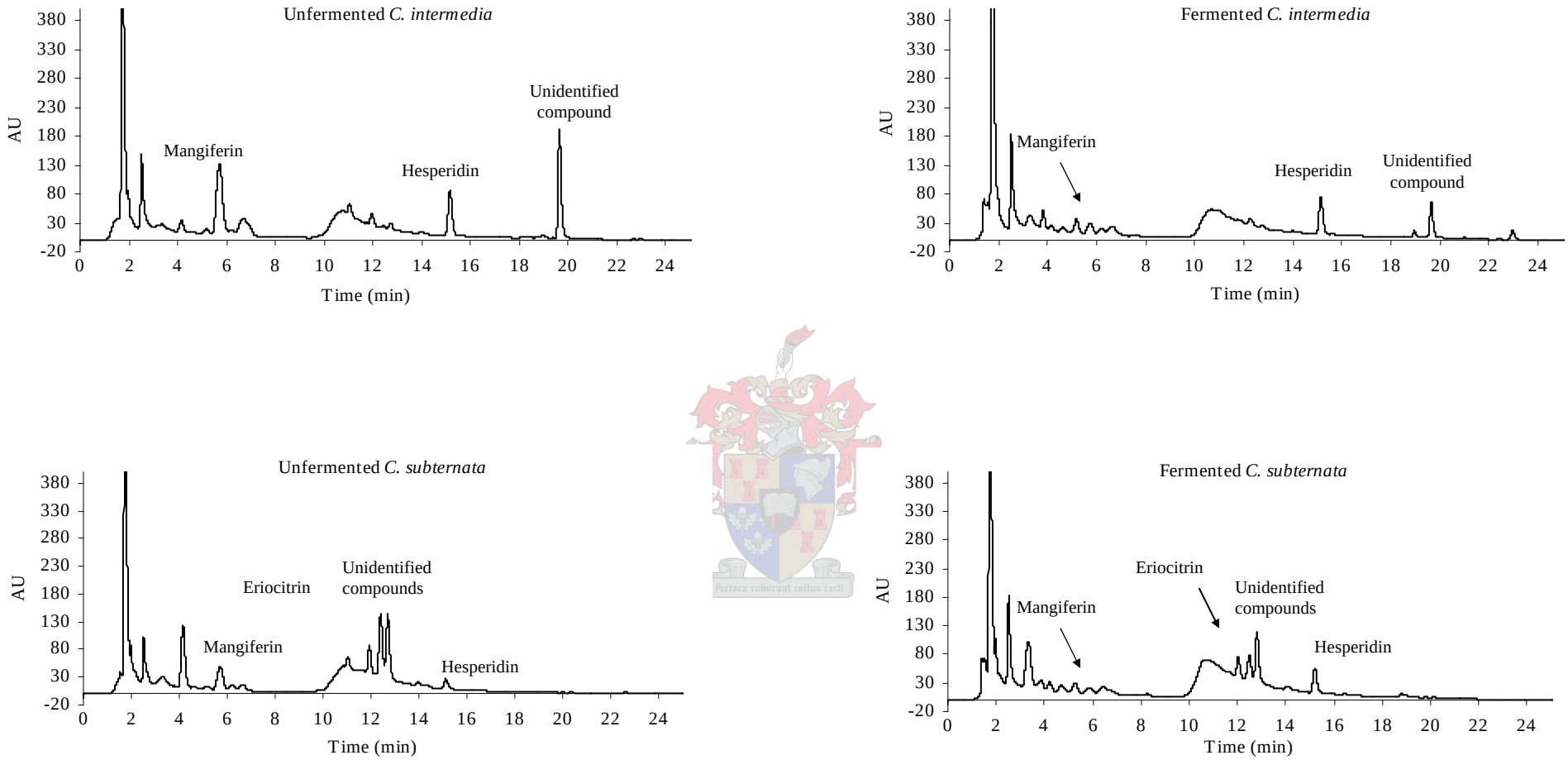


Figure 2 Typical chromatograms of fermented and unfermented *C. intermedia* and *C. subternata* in demonstration of the effect of fermentation on the phenolic composition of *Cyclopia spp.* Detection was at 280 nm.

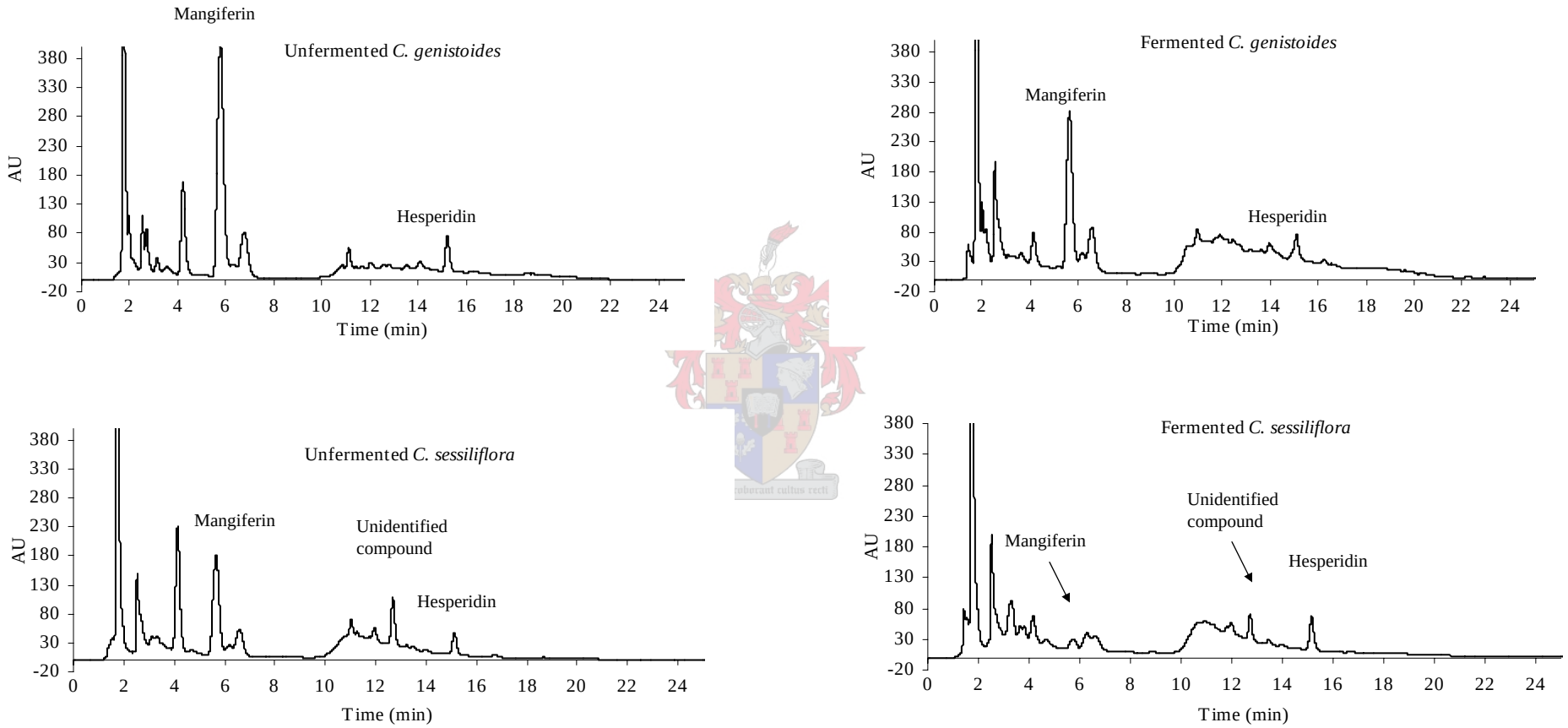


Figure 3 Typical chromatograms of fermented and unfermented *C. genistoides* and *C. sessiliflora* in demonstration of the effect of fermentation on the phenolic composition of *Cyclopia spp.* Detection was at 280 nm.

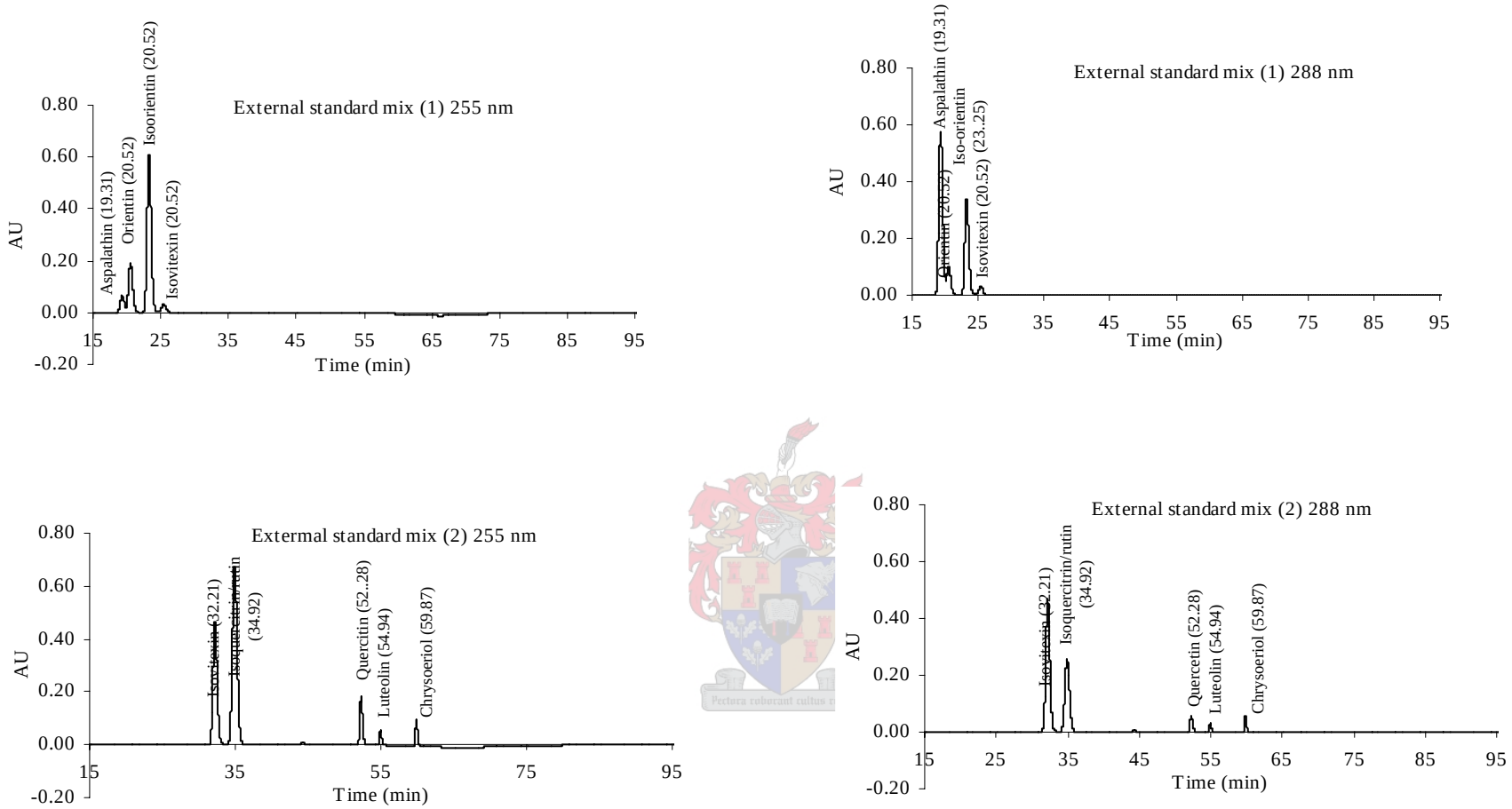


Figure 4 Chromatogram of the 2 standard mixtures of polyphenols used for the quantification of individual compounds in rooibos. The retention time of the compound is given in brackets. Detection was at 255 nm for orientin, iso-orientin, isovitexin, isoquercitrin/rutin, quercetin, luteolin and chrysoeriol and 288 nm for aspalathin and nothofagin.

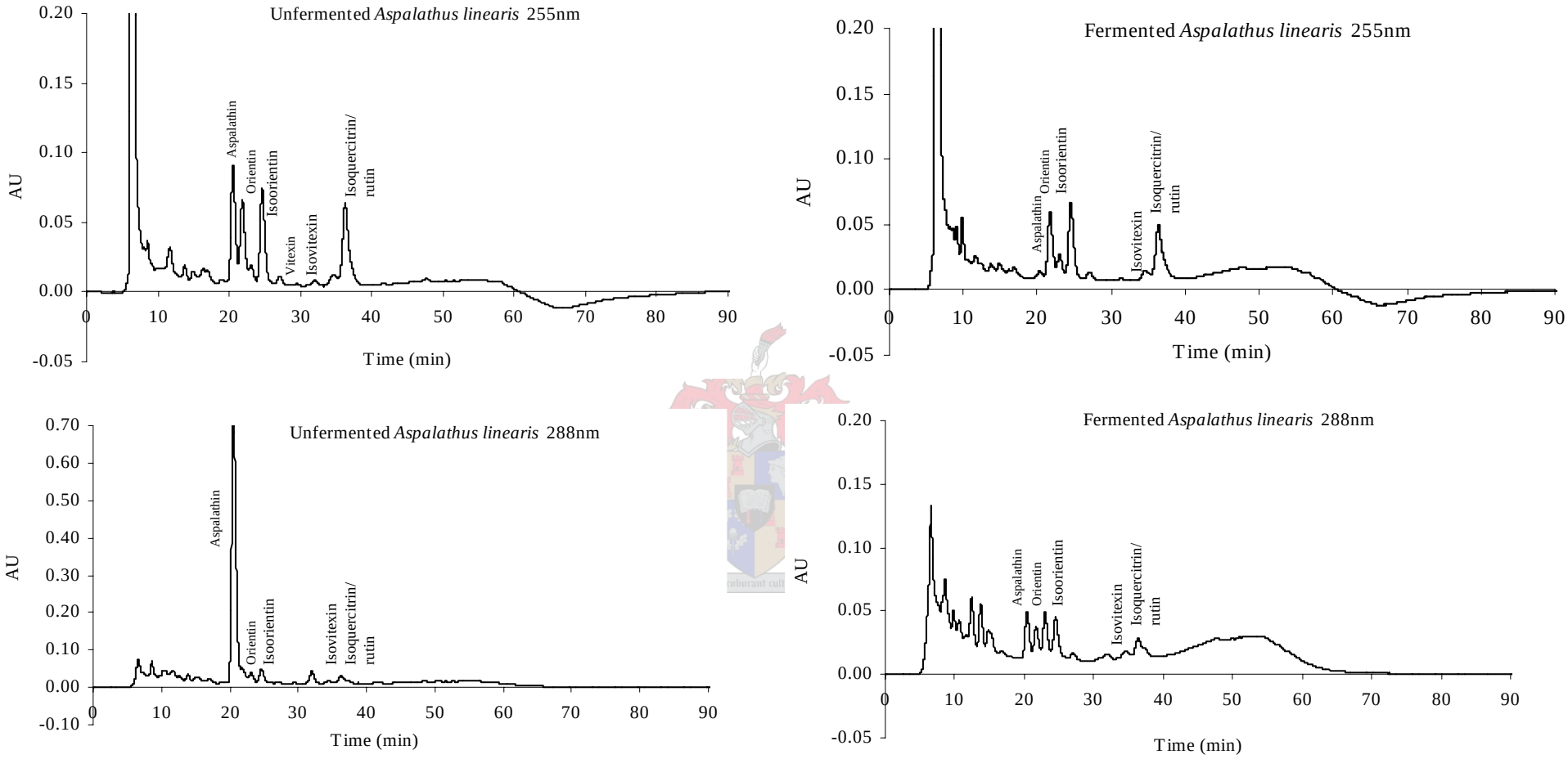


Figure 5 A typical chromatogram of fermented and unfermented rooibos tea, in demonstration of the effect of fermentation on the phenolic composition. Detection at 255 nm and 288 nm.

Table 1 Phenolic content of the aqueous extracts of fermented and unfermented *Cyclopia spp.* in demonstration of variation in levels of compounds in different samples from the same species as determined with reversed-phase HPLC analysis carried out on a Synergy MAX-RP C12 column with acetonitrile and 2% acetic acid in water as eluents.

		XANTHONES		FLAVONE
		Mangiferin	**Isomangiferin**	**Luteolin**
C. intermedia	1	0.20± 0.0004[a]	nd[b]	nd
Fermented	2	0.24± 0.0011	nd	nd
	3	0.30± 0.0047	nd	nd
	4	0.26± 0.0012	nd	nd
	5	0.20± 0.0006	nd	nd
	6	0.18± 0.0012	nd	nd
C.intermedia	1	3.11± 0.0351	nd	nd
Unfermented	2	2.16± 0.0106	nd	nd
	3	2.08± 0.0090	nd	nd
	4	1.81± 0.0440	nd	nd
	5	3.30± 0.0075	nd	nd
	6	2.48± 0.0687	nd	nd
C. subternata	1	0.11± 0.0021	nd	nd
Fermented	2	0.07± 0.0012	nd	nd
	3	0.09± 0.0001	nd	nd
	4	0.05± 0.0013	trace[c]	< 0.01[d]
	5	0.06± 0.0002	trace	< 0.01
	6	0.06± 0.0029	trace	< 0.01
C. subternata	1	1.86± 0.0026	nd	< 0.01
Unfermented	2	0.93± 0.0002	nd	< 0.01
	3	1.32± 0.0014	nd	< 0.01
	4	1.27± 0.0144	nd	< 0.01
	5	1.02± 0.0205	nd	< 0.01
	6	0.72± 0.0196	nd	< 0.01

Table 1 Phenolic content of the aqueous extracts of fermented and unfermented *Cyclopia spp.* (continue).

		XANTHONES		FLAVONE
		Mangiferin	**Isomangiferin**	**Luteolin**
C.genistoides	1	3.91± 0.0303[a]	trace[c]	< 0.01[d]
Fermented	2	2.97± 0.0105	trace	< 0.01
	3	5.85± 0.0213	trace	< 0.01
	4	5.18± 0.0306	trace	< 0.01
	5	4.85± 0.0074	trace	0.011± 0.0030
	6	2.97± 0.0098	trace	0.012± 0.0002
C.genistoides	1	11.33± 0.3591	nd[b]	0.012± 0.0005
Unfermented	2	9.97± 0.0696	nd	0.014± 0.0002
	3	10.10± 0.0332	nd	0.013± 0.0003
	4	9.93± 0.1502	nd	0.013± 0.0017
	5	8.88± 0.0717	nd	0.009± 0.0002
	6	10.04± 0.1132	nd	0.016± 0.0004
C. sessiliflora	1	0.17± 0.0013	trace	nd
Fermented	2	0.18± 0.0005	trace	nd
	3	0.17± 0.0323	trace	nd
	4	0.22± 0.0063	trace	nd
	5	0.15± 0.0014	trace	nd
	6	0.25± 0.0004	trace	nd
C. sessiliflora	1	4.24± 0.0842	nd	< 0.01
Unfermented	2	4.60± 0.0905	nd	< 0.01
	3	4.58± 0.1685	nd	< 0.01
	4	4.85± 0.0318	nd	< 0.01
	5	3.88± 0.0898	nd	< 0.01
	6	4.20± 0.0893	nd	< 0.01

Table 1 Phenolic content of the aqueous extracts of fermented and unfermented *Cyclopia spp.* (continue).

		FLAVANONES				
		Hesperidin	**Hesperetin**	**Eriocitrin**	**Narirutin**	**Eridictyol**
C. intermedia	1	0.53± 0.0033[a]	0.06± 0.0001	nd[b]	0.01± 0.0003	nd
Fermented	2	0.46± 0.0024	0.10± 0.0011	nd	0.02± 0.0002	nd
	3	0.54± 0.0001	0.03± 0.0011	nd	0.02± 0.0002	nd
	4	0.40± 0.0044	0.09± 0.0009	nd	0.02± 0.0003	nd
	5	0.34± 0.0016	0.06± 0.0010	nd	0.01± 0.0001	nd
	6	0.44± 0.0013	0.07± 0.0015	nd	0.02± 0.0012	nd
C.intermedia	1	1.08± 0.0107	nd	nd	0.06± 0.0017	nd
Unfermented	2	1.10± 0.0081	nd	nd	0.04± 0.005	nd
	3	1.14± 0.0023	nd	nd	0.02± 0.0069	nd
	4	1.13± 0.0271	nd	nd	0.03± 0.0010	nd
	5	1.08± 0.0035	nd	nd	0.06± 0.006	nd
	6	1.21± 0.0296	nd	nd	0.03± 0.02	nd
C. subternata	1	0.37± 0.0043	nd	0.29± 0.0016	0.04± 0.0001	nd
Fermented	2	0.18± 0.0001	nd	0.38± 0.0047	0.05± 0.0029	nd
	3	0.38± 0.0001	nd	0.19± 0.0128	0.04± 0.0009	nd
	4	0.24± 0.0010	nd	0.27± 0.0085	0.04± 0.0001	nd
	5	0.26± 0.0062	nd	0.20± 0.0076	0.04± 0.0002	trace[c]
	6	0.21± 0.0120	nd	0.24± 0.0058	0.05± 0.0058	trace
C. subternata	1	0.73± 0.0169	nd	0.46± 0.0055	0.04± 0.0001	nd
Unfermented	2	0.24± 0.0019	nd	0.64± 0.0027	0.06± 0.001	nd
	3	0.43± 0.0071	nd	0.42± 0.0061	0.03± 0.0003	nd
	4	0.39± 0.0006	nd	0.41± 0.0069	0.03± 0.0011	nd
	5	0.46± 0.0012	nd	0.40± 0.0026	0.04± 0.003	nd
	6	0.25± 0.0035	nd	0.52± 0.0099	0.04± 0.0014	nd

Table 1 Phenolic content of the aqueous extracts of fermented and unfermented *Cyclopia spp.* (continue).

		FLAVANONE				
		Hesperidin	**Hesperetin**	**Eriocitrin**	**Narirutin**	**Eridictyol**
C.genistoides	1	0.46± 0.0016[a]	≤ 0.01[d]	nd[b]	nd	nd
Fermented	2	0.45± 0.0069	≤ 0.01	nd	nd	nd
	3	0.47± 0.0074	≤ 0.01	nd	nd	nd
	4	0.47± 0.0059	≤ 0.01	nd	nd	nd
	5	0.47± 0.0036	≤ 0.01	nd	nd	nd
	6	0.49± 0.0039	≤ 0.01	nd	nd	nd
C.genistoides	1	0.93± 0.0278	trace[c]	0.15± 0.0045	nd	nd
Unfermented	2	0.84± 0.0072	trace	0.13± 0.0027	nd	nd
	3	0.82± 0.0054	nd	0.14± 0.0002	nd	nd
	4	0.94± 0.0159	nd	0.12± 0.0052	nd	nd
	5	1.01± 0.0069	nd	0.16± 0.0055	nd	trace
	6	1.01± 0.0013	trace	0.18± 0.0092	nd	nd
C. sessiliflora	1	0.46± 0.0033	nd	0.09± 00068	0.02± 0.0038	trace
Fermented	2	0.48± 0.0067	nd	0.10± 0.0004	0.02± 0.0018	trace
	3	0.47± 0.0043	nd	0.12± 0.0108	0.02± 0.0006	trace
	4	0.40± 0.0005	nd	0.15± 0.0085	0.01± 0.0001	trace
	5	0.44± 0.0020	nd	0.09± 0.0075	0.02± 0.0021	trace
	6	0.43± 0.0012	trace	0.20± 0.0048	0.02± 0.0076	trace
C. sessiliflora	1	0.52± 0.0351	nd	0.26± 0.0114	0.03± 0.0050	trace
Unfermented	2	0.44± 0.0307	nd	0.32± 0.0221	0.02± 0.0003	trace
	3	0.47± 0.0334	nd	0.39± 0.0091	0.03± 0.0015	trace
	4	0.47± 0.0112	nd	0.41± 0.0050	0.03± 0.0016	trace
	5	0.52± 0.0114	nd	0.29± 0.0138	0.03± 0.0005	trace
	6	0.56± 0.0051	nd	0.30± 0.0017	0.03± 0.0001	trace

[a] Values represent the means (% of aqueous extracts) ± SD of two determinations

[b] Not detected.

[c] Trace amounts detected, but levels to low to quantify.

[d] Compounds were detected and quantified, but the concentration was less than 0.01%.

Tabel 2 Quantification of the individual flavonoids in aqueous extracts from fermented and unfermented rooibos tea in demonstration of natural variation in plant material as determined by HPLC on a reversed phase LiChrospher 100 RP–18 C_{18} column with 2% formic acid in water and 100% methanol as eluents.

		DIHYDROCHALCONE	FLAVONES				FLAVONOLS	
		Aspalathin	**Orientin**	**Iso-orientin**	**Vitexin**	**Isovitexin**	**Isoquercitrin/ rutin**	**Quercetin**
Fermented	1	0.66± 0.005[a]	0.69± 0.009	0.81± 0.000	nd	0.26± 0.001	0.27± 0.000	0.01± 0.000
	2	0.61± 0.010	0.72± 0.006	0.90± 0.012	nd	0.21± 0.002	0.29± 0.002	0.02± 0.008
	3	0.58± 0.004	0.61± 0.000	0.74± 0.001	0.09± 0.001	0.25± 0.001	0.24± 0.001	0.01± 0.002
	4	0.36± 0.012	0.71± 0.002	0.89± 0.003	0.11± 0.002	0.20± 0.003	0.30± 0.011	0.02± 0.004
	5	0.37± 0.004	0.76± 0.008	0.94± 0.011	0.11± 0.009	0.21± 0.001	0.32± 0.032	0.01± 0.001
	6	0.82± 0.005	0.72± 0.004	0.86± 0.000	0.11± 0.001	0.24± 0.002	0.35± 0.000	0.02± 0.006
Unfermented	1	7.64± 0.182	0.63± 0.002	0.69± 0.005	0.06± 0.002	< 0.02[b]	< 0.02	< 0.02
	2	8.29± 0.158	0.68± 0.006	0.78± 0.007	0.07± 0.002	< 0.02	< 0.02	< 0.02
	3	8.31± 0.092	0.58± 0.006	0.64± 0.008	0.06± 0.000	< 0.02	< 0.02	< 0.02
	4	6.84± 0.155	0.53± 0.002	0.57± 0.000	0.05± 0.001	< 0.02	< 0.02	< 0.02
	5	3.49± 0.058	0.66± 0.003	0.73± 0.008	0.07± 0.002	< 0.02	< 0.02	< 0.02
	6	6.06± 0.042	0.40± 0.004	0.47± 0.005	0.06± 0.002	< 0.02	< 0.02	< 0.02

[a] Values represent the means (% of aqueous extract) ± SD of two determinations
[b] Trace amounts detected

Addendum B

Table 1 Influence of plant variation on the protective effect of aqueous extracts of fermented *Cyclopia spp*. against AFB_1 induced mutagenesis in the *Salmonella* mutagenicity assay.

		Batches					
Tea	**Concentration**	**1**	**2**	**3**	**4**	**5**	**6**
C. intermedia Fermented	0.5	31.19 ± 12.56[a] abD (281 ± 27)	29.37 ± 17.59 abC (252 ± 27)	14.57 ± 7.09 ef (307± 15)	17.31 ± 4.77 ef (375 ± 12)	17.60 ± 2.77 CDe (350 ± 7)	39.26 ± 1.94 a (312 ± 5)
	1	59.87 ± 9.86 ab (213±24)	49.51 ± 8.65 bc (220± 19)	48.97 ± 11.56 bcd (228± 26)	34.00 ± 2.73 e (326± 5)	41.23 ± 2.75 cde (281± 7)	65.73 ± 2.63 a (234± 4)
	2	81.94 ± 8.77 a (213± 24)	76.44 ± 9.74 a (220±19)	73.88 ± 11.00 a (228±26)	79.60 ± 2.70 a (326± 5)	82.05 ± 10.01 a (281± 7)	81.93 ± 4.15 l a (234± 4)
C. subternata Fermented	0.5	33.47 ± 10.09 bcd (276 ± 21)	39.86 ± 10.21 bc (242 ± 20)	33.36 ± 8.44 bcde (264 ± 18)	25.96 ± 2.63 cde (349 ± 7)	47.66 ± 3.89 ab (262 ± 10)	57.33 ± 2.17 a (259 ± 6)
	1	56.69 ± 7.90 abcde (220±17)	62.85 ± 14.65 ab (189± 30)	61.83 ± 12.21 abc (198± 25)	50.00 ± 10.00 bcde (279± 26)	59.55 ± 4.89 abcd (226± 13)	76.08 ± 8.29 a (203± 22)
	2	82.61± 9.36 a (220±17)	77.84 ± 12.93 bc (189± 30)	69.57± 12.91 bcde (198± 25)	70.74 ± 6.05 bcde (279±26)	77.05 ± 2.39 bcd (226± 13)	96.24 ± 5.48 a (203± 22)
C. genistoides Fermented	0.5	58.19 ± 13.94 acDE (217 ± 30)	42.36 ± 9.30 EfhI (236 ± 19)	29.50 ± 10.21 GI (273 ± 21)	43.54 ± 3.39 aDfGh (298 ± 9)	61.04 ± 0.62 aB (222 ± 2)	46.92 ± 12.82 BcfG (290 ± 34)
	1	79.18 ± 4.37 a (166± 9)	70.43 ± 4.22 aB (172± 9)	64.74 ± 9.56 a (191± 20)	67.03 ± 4.09 c (230± 11)	71.54 ± 8.68 Bd (191± 23)	73.79 ± 5.64 d (210± 15)
	2	95.97 ± 5.68 a (166± 9)	82.98 ± 2.21 cde (172± 9)	85.78 ± 6.60 bcd (191± 20)	81.94 ± 4.32 cde (230± 11)	87.30 ± 5.31 abc (191±23)	94.76 ± 3.98 ab (210± 15)
C. sessiliflora Fermented	0.5	48.93 ± 9.03 a (239 ± 19)	28.94 ± 19.72 abce (267 ± 41)	28.62 ± 10.73 De (275 ± 22)	46.02 ± 5.90 ab (291 ± 15)	61.04 ± 4.78 f (222 ± 13)	41.14 ± 4.68 abcD (307 ± 13)
	1	65.47 ± 6.63 ace (198± 14)	59.90 ± 11.82 bcefg (203± 22)	60.32 ± 5.59 efg (203± 12)	64.90 ± 1.43 Def (238± 2)	69.85 ± 4.09 acD (192± 9)	71.78 ± 3.33 ab (216± 10)
	2	87.88 ± 7.60 abc (196±14)	78.32 ± 6.31 bcde (203± 22)	77.03 ± 9.35 cde (203±12)	84.55 ± 2.97 abcd (238± 2)	93.06 ± 7.14 a (192±9)	92.56 ± 5.07 a (216±10)

[a] Values represent the mean ± S.D of percentage inhibition followed by the revertant count in brackets. Means were calculated for six samples with five replications. Means in the same column followed by the same letter are not significantly different ($P < 0.05$) and capital letters indicate $P < 0.1$

Table 2 Influence of plant variation on the protective effect of aqueous extracts of fermented rooibos tea and black, oolong and green tea against AFB_1 induced mutagenesis in the *Salmonella* mutagenicity assay.

		Batches					
Tea	**Concentration**	**1**	**2**	**3**	**4**	**5**	**6**
Rooibos tea Fermented	0.1	12.30 ± 9.63[a] abcd (335 ± 19)	9.63 ± 6.22 abcde (341 ± 13)	18.98 ± 2.28 abc (318 ± 5)	-2.11 ± 4.75 d (370 ± 11)	23.68 ± 13.36 ae (307 ± 30)	22.71 ± 7.92 ab (309 ± 18)
	0.5	88.21 ± 5.02 a (145 ±11)	79.32 ± 10.31 ab (151 ±21)	82.59 ± 7.00 ab (150± 15)	71.77 ± 3.37 b (216± 9)	84.15 ± 3.86 a (154± 10)	89.65 ± 3.30 a (162± 9)
	1	97.49 ± 5.63 ab (123± 12)	91.61 ± 4.49 b (123± 9)	92.16 ± 2.95 b (127± 6)	90.38 ± 5.39 b (162± 14)	92.21 ± 5.15 b (130± 14)	100.87 ± 1.19 a (129± 3)
	2	107.36 ± 4.78 a (123± 12)	91.35 ± 3.61 a (123±9)	96.21 ± 8.45 b (127±6)	91.41 ± 2.83 bc (162±14)	98.64 ± 0.86 c (130± 14)	103.76 ± 3.14 c (129±3)
Black tea Fermented	0.1	36.82 ± 14.85 a (274 ± 33)	35.52 ± 6.14 a (277 ± 13.5)	28.99 ± 11.09 a (294 ± 23.7)	37.79 ± 12.57 a (272 ± 28)	44.20 ± 6.85 a (256 ± 15)	45.01 ± 8.11 a (254 ± 18)
	0.5	102.51 ± 5.20 a (111 ± 11)	102.51 ± 2.86 a (98 ± 6)	87.67 ± 5.21 c (138 ± 11)	91.28 ± 4.36 bc (159 ± 11)	95.60 ± 1.48 abc (120 ± 4)	96.04 ± 3.92 ab (143± 10)
	1	97.99 ± 7.22 a (121± 15)	104.42 ± 5.77a (94± 12)	98.53 ± 3.78 a (113± 8)	95.40 ± 3.81 a (147± 10)	99.66 ± 5.59 a (108± 14)	101.68 ± 5.39 a (127± 14)
	2	101.25 ± 6.19 a (121± 15)	99.63 ± 6.04 a (94± 12)	94.83 ± 6.25 a (113± 8)	97.05 ± 4.53 a (147± 10)	98.31± 0.73 a (108±14)	101.68 ± 4.87 a (127± 14)
Oolong tea Semi-fermented	0.1	47.28 ± 4.73 a (248 ±10)	25.75 ± 8.73 bcde (302 ±19)	26.28 ± 9.28 bcd (300 ± 21)	32.64 ± 9.91 ab (285 ± 21)	28.52 ± 4.34 bc (295 ± 9)	24.73 ± 7.35 bcde (304± 15)
	0.5	92.98 ± 8.39 a (133 ± 18)	86.99 ± 4.23 a (134 ± 9)	87.07 ± 2.54 a (139 ± 5)	89.35 ± 6.42 a (165 ± 17)	83.27 ± 2.81 a (156 ± 7)	92.27 ± 4.73 a (155 ± 13)
	1	94.15 ± 5.20 a (131± 11)	98.50 ± 8.12 a (107± 17)	98.62 ± 4.94 a (112± 10)	93.68 ± 2.12 a (152± 6)	98.17 ± 4.07 a (112± 11)	100.27 ± 5.55 a (131± 15)
	2	101.40 ± 6.43 aCD (131± 11)	94.33 ± 6.59 abef (107± 17)	93.79 ± 4.59 Cefg (112± 10)	93.61 ± .54 aDefg (152± 6)	96.88 ± 3.12 be (112± 11)	103.23 ± 2.18 ab (131± 15)
Green tea Unfermented	0.1	44.28 ± 15.35 aB (256 ±34)	27.58 ± 9.89 Bcd (297 ± 22)	35.69 ± 4.18 ac (277 ± 9)	22.87 ± 6.66 de (309 ± 15)	48.66 ± 5.59 a (245 ± 12)	24.82 ± 10.78 cde (304 ± 24)
	0.5	84.11 ± 10.36 a (155 ±22)	93.27 ± 8.67 a (113 ± 14)	85.78 ± 5.18 a (147 ± 5)	67.10 ± 6.15 a (230 ± 16)	71.68 ± 18.92 a (191 ± 50)	90.78 ± 5.90 a (159 ± 15)
	1	94.06 ± 4.20 abc (131± 10)	102.07 ± 6.49 a (102± 14)	93.10 ± 7.49 abcd (128± 16)	79.12 ± .36 e (195± 7)	89.63 ± 1.35 bcd (138± 4)	96.64 ± 2.98 ab (140± 8)
	2	99.92 ± 4.50 a (131± 10)	98.41 ± 3.36 a (102± 14)	91.72 ± 11.53 a (128± 16)	95.33± 3.91 a (195± 7)	98.04 ± 6.41 a (138± 4)	103.48 ± 4.26 a (140± 8)

[a] Values represent the mean ± S.D of percentage inhibition followed by the revertant count in brackets. Means were calculated for six samples with five replications. Means in the same column followed by the same letter are not significantly different ($P < 0.05$) and capital letters indicate $P < 0.1$

Table 3 Influence of plant variation on the protective effect f aqueous extracts of unfermented *Cyclopia spp*. and rooibos tea against AFB_1 induced mutagenesis in the *Salmonella* mutagenicity assay.

		Batches					
Sample	**Concentration**	**1**	**2**	**3**	**4**	**5**	**6**
C. intermedia Unfermented	0.5	76.00 ± 4.11[a] cde (174 ± 9)	76.71 ± 9.67 bcd (157 ± 20)	74.66 ± 7.92 cdef (168 ± 16)	70.26 ± 1.45 af (220 ± 4)	79.54 ± 5.71 Ac (167 ± 15.)	85.76 ± 2.70 Ab (174 ± 7)
	1	97.99 ± 3.15 ab (121± 7)	98.23 ± 3.57 a (108± 7)	86.64 ± 8.82 bde (140± 18)	87.77 ±4.75 cde (169± 12)	93.39 ± 1.37 abcd (127±4)	94.89 ± 3.15 abc (147±8)
	2	100.75 ± 4.72 ab (121± 7)	96.32 ± 4.20 abc (108± 7)	92.67 ± 4.35 c (140± 18)	93.75± 1.94 bc (169± 12)	98.58 ± 3.29 abc (127± 4)	101.61± 3.25 a (147± 8)
C. subternata Unfermented	0.5	64.63 ± 8.70 c (201 ± 19)	78.88 ± 5.28 ab (152 ± 11)	71.38 ± 9.89 bc (176 ± 21)	61.13 ± 7.71 c (247 ± 20)	86.25 ± 4.85 d (148 ± 13)	79.71 ± 2.23 ab (192 ± 6)
	1	93.48 ± 1.81 b (134± 3)	93.88 ± 3.56 b (116± 6)	89.57 ± 9.98 a (138± 20)	81.39 ± 3.74 ab (188± 11)	88.69 ± 2.74 a (142± 7)	95.90 ± 3.68 b (148± 10)
	2	98.75 ± 4.25 ab (220± 17)	95.79 ± 4.69 abcde (189± 30)	97.84 ± 4.77 abc (198± 25)	90.80 ± 1.98 bcde (279± 26)	99.66 ± 5.28 a (226± 13)	97.04± 3.17 abcd (203± 22)
C. genistoides Unfermented	0.5	45.07 ± 6.87 bde (248 ± 14)	35.39 ± 6.47 de (252 ± 13)	49.48 ± 8.00 abc (226 ± 17)	48.25 ± 3.97 abcd (285 ± 10)	60.57 ± 5.06 a (223 ± 13)	55.85 ± 5.68 ab (263 ± 15)
	1	78.51 ± 12.26 ab (168± 26)	56.96 ± 12.25 Def (203± 24)	51.64 ± 11.20 ef (221±23)	62.91 ± 2.83 Ce (242± 7)	79.61 ± 3.47 a (167± 9)	70.84 ± 7.12 bC (218± 19)
	2	84.11 ± 6.09 abc (166± 9)	78.10 ± 6.47 bcde (172± 9)	70.43 ± 10.63 cde (191± 20)	82.42 ± 6.32 abcd (230± 11)	92.82 ± 5.56 a (191± 23)	90.53 ± 5.22 ab (210± 15)
C. sessiliflora Unfermented	0.5	78.18 ± 16.63 ab (239 ± 19)	79.15 ± 10.45 a (267 ± 40)	68.94 ± 13.37 abcd (275 ± 22)	63.19 ± 7.23 bde (291 ± 15)	64.91 ± 2.19 bde (222 ± 13)	75.81 ± 3.94 abc (307 ± 12)
	1	98.83 ± 2.32 a (119±5)	97.54 ± 3.28 ab (109± 7)	81.47 ± 4.00 cd (152± 8)	84.05 ± 2.11 cd (180± 5)	85.04 ± 1.96 c (150± 5)	97.25 ± 5.05 ab (140± 13)
	2	101.00 ± 2.26 abd (119±5)	93.35 ± 2.07 ef (109± 7)	94.41 ± 5.53 abde (152± 8)	94.78± 5.39 def (180± 5)	102.64± 2.13 a (150± 5)	102.62 ± 2.48 ab (140± 13)
Rooibos tea Unfermented	0.5	61.04 ± 6.90 bc (210 ±15)	58.40 ± 9.63 bcd (199 ±20)	52.16± 13.20 cde (220 ±27)	56.80± 6.40 cde (260 ±17)	73.04 ± 5.20 ab (187± 14)	85.34 ± 7.51 a (175 ±19)
	1	88.63 ± 10.63 abc (148±24)	82.02 ± 10.44 bc (136± 12)	62.50±3.37 d (196± 8)	76.24 ± 4.28 c (200± 11)	94.24 ± 5.99 ab (127± 17)	99.80 ± 1.89 a (134± 4)
	2	92.47 ± 6.22 abcd (148± 24)	96.32 ± 7.75 ab (136±12)	85.17± 6.24 bcde (196± 8)	89.42 ± 6.54 bcde (200± 11)	96.19 ± 4.70 ac (127± 17)	102.49± 4.65 a (134± 4)

[a] Values represent the mean ± S.D of percentage inhibition followed by the revertant count in brackets. Means were calculated for six samples with five replications. Means in the same column followed by the same letter are not significantly different ($P < 0.05$) and capital letters indicate $P < 0.1$

Addendum C

Method development for investigating the effect of unfermented *Cyclopia spp.*, *Aspalathus linearis* and *Camellia sinensis* on rat hepatic cytochrome P450 stability

Methods

Sample preparation

Procedures for sample preparation and determination of cytochrome P450 and protein content were as described in Chapter 4.

Method for assessment of stability of cytochrome P450 in the rat liver homogenate

The liver homogenate fraction (S9) was diluted to a protein concentration of 2 mg mL^{-1} with a Tris-HCL buffer (50 mM, pH 7.4). Samples consisted of 9 mL of diluted S9 and 1 mL of buffer or dissolved tea extract incubated at 37°C for 15 min. Half of the sample volume, used for the 0 h reading, was placed on ice and bubbled with carbon monoxide for 1 min. The sample was divided between a sample and reference cell (2.5 ml in each) and the baseline recorded with a wavelength scan from 380 to 500 nm, except if stated otherwise. A few grains of solid sodium dithionite were added to the sample cell and the difference spectra were recorded after proper mixing. During this step the carbon monoxide binding to reduced cytochrome P450 was monitored.

The procedure was repeated after 4 hrs of incubation at 37°C with the remaining 5 mL of every sample. The cytochrome P450 content and the difference between the absorbance at 450 nm and 425 nm ($\Delta A_{450-425}$) were calculated for 0 and 4 h, to monitor the breakdown of cytochrome P450. The cytochrome P450 content was calculated using the same formula as for the determination of cytochrome P450 content (equation 1, Chapter 4) and the percentage change in $\Delta A_{450-425}$ was calculated according to the formulas below.

$$\% \text{ Change } \Delta A_{450-425} = [(\Delta A_{450-425\ 0\,h} - \Delta A_{450-425\ 4\,h}) / \Delta A_{450-425\ 0\,h}]*100 \qquad \text{[equation 2]}$$

$$\Delta A_{450-425} = \text{Absorption at 450 nm} - \text{Absorption at 425 nm} \qquad \text{[equation 3]}$$

Preliminary studies

(i) Stability of cytochrome P450 in the rat liver homogenate and the microsomal fractions

The stability of cytochrome P450 was firstly investigated in the microsomal fraction. The procedure described for the assessment of stability of cytochrome P450 in the rat liver homogenate fraction, was followed with the following exceptions: microsomes were diluted to a protein concentration of 1 mg protein mL^{-1} and the stability of cytochrome P450 was monitored at 15 min intervals over a 90 min period as well as 1 h intervals over a 4 h period.

The stability of cytochrome P450 in the rat liver S9 fraction (2 mg proteing mL^{-1}) was monitored at 1 h intervals over a 4 h period.

(ii) Measurement of cytochrome P450 stability and possible stabilising by unfermented C. intermedia

Reproducibility of measuring cytochrome P450 breakdown in the S9 fraction and the possible stabilising effect of an aqueous tea extract were monitored over 4 hrs at 37˚C. An aqueous extract of randomly selected unfermented *C. intermedia* was used for preparation of a stock solution of 0.4 % (w/v). The stock solution was added in 1 mL quantities to 9 mL of the diluted S9 fraction, while control measurements contained 1 mL of buffer and 9 mL of S9 fraction. The cytochrome P450 content (equation 1), $\Delta A_{450-425}$ (equation 3), the percentage change in $\Delta A_{450-425}$ (equation 2) and percentage change in cytochrome P450 content (equation 4) were calculated.

$$\% \text{ Change in cytochrome P450} = [(\text{P450 content}_{0\,h} - \text{P450 content}_{4\,h}) / \text{P450 content}_{0\,h}] * 100 \quad \text{[equation 4]}$$

Results and Discussion

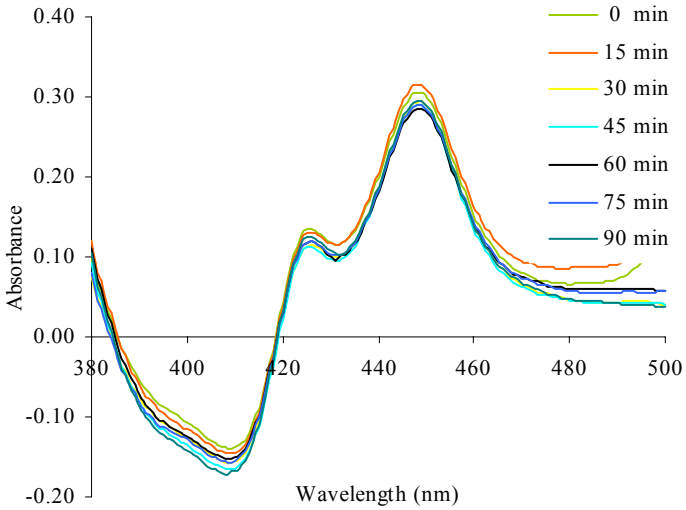
Difference spectra of carbon monoxide (CO) saturated, dithionite-reduced rat liver microsomes, incubated at 37˚C, showed no change over 90 min when recorded at 15 min intervals (Fig. 1a), while relative small changes were evident after 4 hrs when recorded at hourly intervals (Fig. 1b). The peak at 450 nm decreased in the difference spectra of CO-saturated, dithionite-reduced S9 while the peak at 425 nm noticeably increased, indicating reduced stability of cytochrome P450 over 4 hrs (Fig. 2). It was consequently decided to use rat liver S9 to monitor the stabilising effect of different teas and selected phenolic compounds.

A decrease in cytochrome P450 stability and cytochrome P450 concentration was significantly counteracted by unfermented *C. intermedia*, demonstrated by the significantly ($P < 0.001$) smaller values in the % change in cytochrome P450 concentration (nM mg^{-1} protein) and % change in $\Delta A_{450\text{-}A425}$ than for that of the control samples (Table 1). The cytochrome P450 concentration of rat liver S9 was significantly ($P < 0.001$) reduced in the presence and absence (control) of *C. intermedia* after 4 hrs (Table 1). *C. intermedia*, however, counteracted the decrease in cytochrome P450 content in the control sample at 4 hrs which was significantly ($P < 0.001$) less at 0.265 nM cytochrome P450 mg^{-1} protein than that of the sample at 0.497 nM cytochrome P450 mg^{-1} protein. The same trend was observed when calculating the difference in absorbance at 450 nm and 425 nm ($\Delta A_{450\text{-}A425}$, Table 1) as a measure of change of cytochrome P450 stability.

Conclusion

Due to interference (cause unknown) and erratic absorption values at 490 nm, resulting in variation in the cytochrome P450 concentration calculated, not reflected in the difference spectra, it was decided that the decrease in cytochrome P450 stability in the S9 fraction would be calculated as the % change in $\Delta A_{450\text{-}425}$ in subsequent experiments. The % change in $\Delta A_{450\text{-}425}$ was also more sensitive for monitoring cytochrome P450 stability than determination of the enzyme concentration (nM mg^{-1} protein). A concentration of 0.02% (w/v) was selected for studies on the teas since higher concentrations (0.04%) interfered with recording of difference spectrum for some of the *Cyclopia spp.* (data not shown).

a)



b)

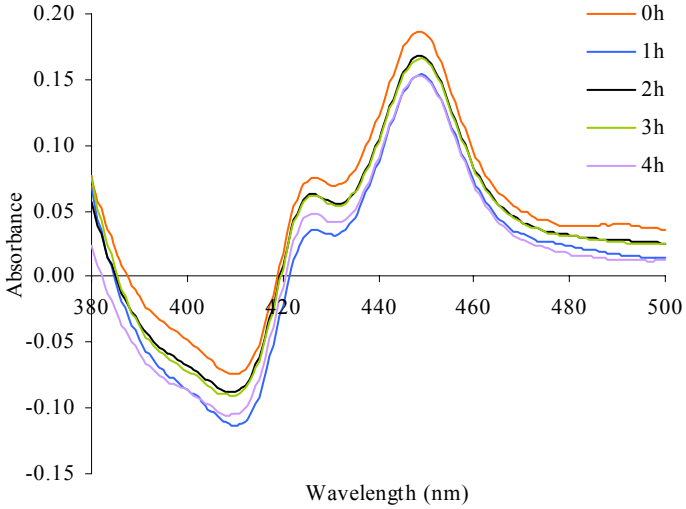


Figure 1 Difference spectra of CO-saturated, dithionite-reduced rat liver microsomes (2.079 nM P450 mg^{-1} protein, 1 mg protein mL^{-1}) incubated at 37°C recorded every a) 15 min for 90 min and b) every h for 4 hrs.

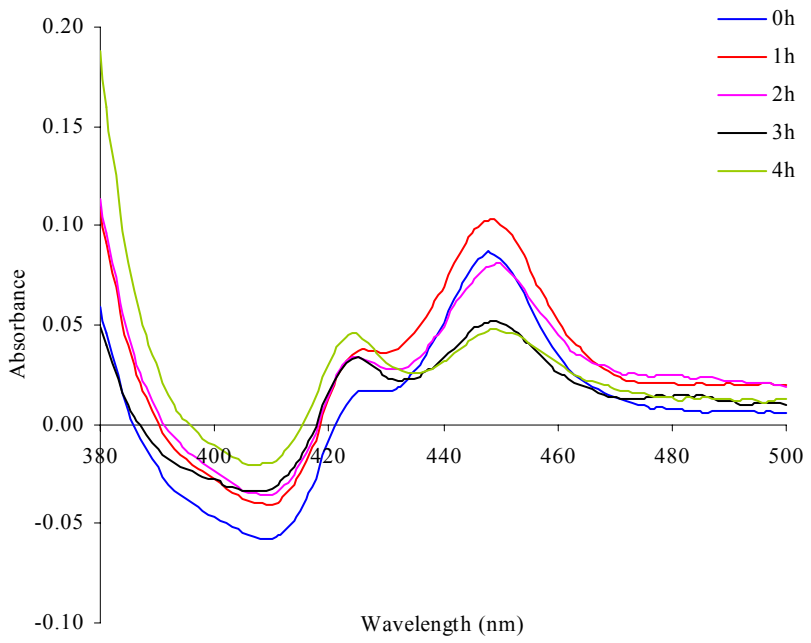


Figure 2 Difference spectra of CO-saturated, dithionite-reduced rat liver S9 fractions (0.625 nM P450 mg^{-1} protein, 2 mg protein mL^{-1}) at 37°C, recorded every h for 4 hrs.

Table 1 Preliminary study on the stability of cytochrome P450 in rat liver S9 fractions (2 mg protein mL^{-1}) over 4 hrs at 37˚C monitored by (i) cytochrome P450 concentration and (ii) $\Delta A_{450\text{-}A425}$ in the absence and presence of unfermented *C. intermedia*.

	Calculation	Control[a]	Sample[a]
0 h	P450 (nM mg^{-1} protein)[b]	0.625 ± 0.030	0.597 ± 0.018
	$\Delta A_{450\text{-}A425}$[c]	0.089 ± 0.007	0.111 ± 0.003
4 h	P450 (nM mg^{-1} protein)	0.265 ± 0.027	0.497 ± 0.016
	$\Delta A_{450\text{-}A425}$	0.023 ± 0.003	0.088 ± 0.004
	% change in P450[c]	57.38 ± 5.69	16.75 ± 3.79
	% change in $\Delta A_{450\text{-}A425}$[c]	73.60 ± 4.28	20.89 ± 3.03

[a] Values represent the mean ± S.D. of five assay repeats.

[b] The P450 content was calculated as described for P450 determination, except after addition of tea solution (sample) or buffer (control):

$$\text{nM P450 mg}^{-1}\text{ protein} = (A_{450}\text{-}A_{490})*1000/91*1/[\text{mg protein mL}^{-1}] \quad \text{[equation 1]}$$

[c] The percentage change in cytochrome P450 content (equation 4) and in $\Delta A_{450\text{-}A425}$ (equation 3) the % change in $\Delta A_{450\text{-}A425}$ (equation 2) after 4 hrs of incubation at 37ºC.

[d] Statistical analysis was performed on the data and is discussed in the text.