EVALUATION OF ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF HONEYBUSH TEA (*CYCLOPIA*)

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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

In vitro model systems, including the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) radical scavenging methods and inhibition of linoleic acid peroxidation, were used to assess the antioxidant and antiradical potential of honeybush aqueous extracts and crude phenolic fractions and compounds. For the purpose of evaluating test materials of this divergent nature in the same assay, comparison of antiradical efficiencies was executed on a mass basis. In addition, the comparison of the antiradical efficiencies of the phenolic compounds was performed on a molar basis to elucidate structure-activity relationships.

Comparison of aqueous extracts prepared from unfermented and fermented material of *Cyclopia intermedia*, *C. genistoides*, *C. maculata*, *C. sessiliflora* and *C. subternata* showed that the extract of unfermented *C. sessiliflora* (highest total polyphenol content) was the most effective scavenger of both $DPPH^{\bullet}$ and $O_2^{\bullet-}$ ($P \leq 0.05$). Fermented *C. subternata*, followed by fermented *C. maculata* and *C. intermedia*, showed the weakest hydrogen-donating (H-donating) ability towards $DPPH^{\bullet}$, whilst the latter two species showed the weakest scavenging of $O_2^{\bullet-}$ ($P \leq 0.05$). This phenomenon is partially attributed to the soluble solids of fermented *C. subternata*, which did not differ significantly from fermented *C. maculata* and *C. intermedia*, containing the lowest total polyphenol contents ($P \leq 0.05$). Aqueous extracts from fermented and unfermented *C. genistoides* showed the most effective inhibition of linoleic acid peroxidation, with unfermented *C. intermedia* showing the weakest activity.

Fermentation lowered the antiradical efficiency of aqueous extracts from the different species and crude phenolic fractions from *C. intermedia* towards $DPPH^{\bullet}$ and $O_2^{\bullet-}$, possibly due to the oxidation of the phenolic compounds. However, inhibition of linoleic acid peroxidation by aqueous extracts and phenolic fractions were not affected by fermentation ($P \geq 0.05$), except for the aqueous extract of unfermented *C. intermedia* being slightly less effective than its fermented equivalent ($P \leq 0.05$).

A strong relationship was observed between the structures of honeybush tea phenolic compounds and their relative antiradical efficiencies. With comparison on a molar basis, the reference compound, quercetin, as well as the honeybush polyphenols, luteolin, mangiferin and isomangiferin, were the most effective scavengers of $DPPH^{\bullet}$,

whereas hesperidin showed the weakest activity. Formononetin, flemichapparin, medicagol, 4-coumaric acid and naringenin were ineffective towards DPPH$^{\bullet}$ at the concentrations tested. For the $O_2^{\bullet-}$ scavenging assay, the reference compound quercetin was the most effective compound on a molar basis. Of the honeybush polyphenols, luteolin and eriodictyol were the most effective scavengers of $O_2^{\bullet-}$, with activity comparable to that of the antioxidant enzyme, superoxide dismutase (SOD), whereas hesperetin was the least effective. Formononetin, flemichapparin, medicagol, 4-coumaric acid and the synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), were ineffective as scavengers of $O_2^{\bullet-}$ at the concentrations tested. The excellent antiradical activity of luteolin is attributable to the presence of the 3',4'-dihydroxy configuration in the B-ring in combination with the 5-OH and 4-carbonyl groups in the A- and C-rings.

Aqueous extracts from *C. maculata* (*ex* Du Toitskloof) were used to quantify the effect of particle size and extraction time on the antiradical activity of the soluble solids towards DPPH$^{\bullet}$ and $O_2^{\bullet-}$. Aqueous extracts prepared from tea material with a particle size $\leq$ 2 mm were more effective scavengers of DPPH$^{\bullet}$ and $O_2^{\bullet-}$ than aqueous extracts prepared from tea material with a particle size > 2 mm (containing more woody material). The weaker activity of the latter extracts was attributable to the soluble solids containing less total polyphenols. With increasing extraction times, the total polyphenol content of the soluble solids increased, which explains the increase in the activity of aqueous extracts to scavenge DPPH$^{\bullet}$ and $O_2^{\bullet-}$.

Results presented in this study thus clearly demonstrated the *in vitro* antioxidant and antiradical potential of honeybush tea aqueous extracts, crude phenolic fractions and phenolic compounds. Follow-up studies using other *in vitro*, as well as *ex vivo* methods are advisable to fully understand the antioxidant and antiradical properties of honeybush tea and its potential role as a functional beverage.

Opsomming

In vitro toetssisteme, onder andere neutralisering van die 1,1-difeniel-2-pikrielhidrasiel ($DPPH^{\bullet}$) en superoksied anioon ($O_2^{\bullet-}$) radikale, asook inhibisie van linoleïensuur oksidasie, is aangewend om die antioksidant- en antiradikaal-potensiaal van waterekstrakte, fenoliese fraksies en fenoliese verbindings van heuningbostee te bepaal. Die evaluering van dié uiteenlopende tipes toetsmateriaal in dieselfde studie, het die vergelyking van die antiradikaal-aktiwiteit op 'n massa basis, genoodsaak. Die antiradikaal-aktiwiteit van suiwer fenoliese verbindings is egter ook op 'n molêre basis vergelyk in 'n poging om hul struktuur-aktiwiteitsverwantskap te verklaar.

Waterekstrakte van ongefermenteerde en gefermenteerde materiaal van vyf verskillende spesies, insluitend *Cyclopia intermedia*, *C. genistoides*, *C. maculata*, *C. sessiliflora* en *C. subternata*, is op grond van antioksidant- en antiradikaal-aktiwiteit vergelyk. Die waterekstrak van ongefermenteerde *C. sessiliflora* (hoogste totale polifenolinhoud) het die mees effektiewe neutralisering van $DPPH^{\bullet}$ en $O_2^{\bullet-}$ ($P \leq 0.05$) getoon. Gefermenteerde *C. subternata*, gevolg deur gefermenteerde *C. maculata* en *C. intermedia*, het die swakste vermoë getoon om waterstof (H) aan $DPPH^{\bullet}$ te doneer. Gefermenteerde *C. maculata* en *C. intermedia* het ook die swakste potensiaal getoon om $O_2^{\bullet-}$ te neutraliseer ($P \leq 0.05$). Dit kan waarskynlik toegeskryf word aan die lae totale polifenolinhoud van die opgeloste vaste stowwe in die waterekstrakte van hierdie plant materiaal. Waterekstrakte wat berei is van die gefermenteerde en ongefermenteerde materiaal van *C. genistoides*, het linoleïensuur oksidasie die beste geïnhibeer, terwyl ongefermenteerde *C. intermedia* die swakste aktiwiteit getoon het.

Fermentasie het die effektiwiteit van waterekstrakte van verskillende spesies, asook van fenoliese fraksies van *C. intermedia*, om $DPPH^{\bullet}$ and $O_2^{\bullet-}$ te neutraliseer, verminder. Dit kan moontlik toegeskryf word aan die oksidasie van die fenoliese verbindings tydens die fermentasieproses. Daar was egter geen beduidende verskille in die mate van linoleïensuur oksidasie tussen waterekstrakte en fenoliese fraksies se gefermenteerde en ongefermenteerde ekwivalente nie ($P \geq 0.05$). 'n Uitsondering was die waterekstrak van ongefermenteerde *C. intermedia* wat ietwat minder effektief was as die gefermenteerde ekwivalent ($P \leq 0.05$).

'n Baie duidelike verwantskap is tussen die onderskeie strukture van heuningbos

fenoliese verbindings en hul relatiewe antiradikaal-aktiwiteite, waargeneem. Die verwysingsverbinding, kwersetien, asook die heuningbostee fenole, luteolien, mangiferien en isomangiferien, was die mees effektiewe neutraliseerders van DPPH$^{\bullet}$ op 'n molêre basis. Hesperidien, daarenteen, het die swakste aktiwiteit teenoor hierdie radikaal getoon, terwyl formononetien, flemichapparien, medicagol, 4-koumaarsuur en naringenien onaktief was. Kwersetien het ook die beste neutralisering van $O_2^{\bullet-}$ getoon. Die heuningbostee fenole, luteolien en eriodictyol, het die beste antiradikaal-aktiwiteit teenoor $O_2^{\bullet-}$ getoon, soortgelyk aan dié van die antioksidant ensiem, superoksied dismutase (SOD). Hesperetien het die swakste inhibisie van $O_2^{\bullet-}$ getoon, terwyl formononetien, flemichapparin, medicagol, 4-koumaarsuur, en die sintetiese antioksidante, gebutileerde hidroksietolueen (BHT) en gebutileerde hidroksieanisool (BHA), oneffektief was. Die uitstekende antiradikaal-aktiwiteit van luteolien kan aan die teenwoordigheid van die 3',4'-dihidroksie konfigurasie in die B-ring, tesame met die 5-OH en 4-karboniel groepe in onderskeidelik die A- en C-ring toegeskryf word.

Die effek van partikelgrootte en ekstraksietyd op die vermoë van opgeloste vaste stowwe om DPPH$^{\bullet}$ en $O_2^{\bullet-}$ te neutraliseer, is gekwantifiseer deur van waterekstrakte van *C. maculata* (*ex* Du Toitskloof) gebruik te maak. Waterekstrakte wat voorberei is van tee materiaal met 'n snitgrootte $\leq$ 2 mm, het meer effektiewe neutralisasie van DPPH$^{\bullet}$ en $O_2^{\bullet-}$ teweeggebring as waterekstrakte soos berei van tee materiaal met snitgroottes > 2 mm (met 'n hoër persentasie houtagtige materiaal). Die opgeloste vaste stowwe van laasgenoemde ekstrakte bevat minder totale polifenole, wat waarskynlik die swakker aktiwiteit daarvan verklaar. Die totale polifenolinhoud van die opgeloste vaste stowwe neem toe soos wat die ekstraksietyd verleng word. Dié waarneming verklaar die toename in die antiradikaal-aktiwiteit van die waterekstrakte teenoor DPPH$^{\bullet}$ en $O_2^{\bullet-}$ dienooreenkomstig met die toename in die ekstraksietyd.

Die *in vitro* antioksidant- en antiradikaal-potensiaal van heuningbos waterekstrakte, fenoliese fraksies en fenoliese verbindings, is duidelik in hierdie studie aangetoon. Verdere *in vitro*, sowel as *ex vivo* metodes, is nodig om die antioksidant- en antiradikaal-aktiwiteit van heuningbostee ten volle te verstaan, asook om die potensiaal van die tee as 'n funksionele drankie te ontwikkel.

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Abbreviations

AAPH	=	2,2'-azobis(2-amidinopropane) hydrochloride
$ABTS^{\bullet+}$	=	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation
ANOVA	=	analysis of variance
AO	=	antioxidant
AR	=	analytical reagent
BHA	=	butylated hydroxyanisole
BHT	=	butylated hydroxytoluene
CD	=	conjugated diene
CHD	=	coronary heart disease
DMSO	=	dimethyl sulphoxide
$DPPH^{\bullet}$	=	1,1-diphenyl-2-picrylhydrazyl radical
EDTA	=	ethylenediamine-tetraacetic acid disodium salt
EGC	=	epigallocatechin
EGCG	=	epigallocatechin gallate
ESR	=	electron spin resonance
EtOH	=	ethanol
FM	=	fermented methanol fraction
FD	=	fermented diethyl ether fraction
GAE	=	gallic acid equivalents
Gen(F)	=	fermented *C. genistoides*
Gen(U)	=	unfermented *C. genistoides*
H	=	hydrogen
HCl	=	hydrochloric acid
H_2O	=	water
H_2O_2	=	hydrogen peroxide
HPLC	=	high performance liquid chromatography
IC_{50}	=	[AO] giving 50% inhibition
Int(F)	=	fermented *C. intermedia*
Int(U)	=	unfermented *C. intermedia*
KOH	=	potassium hydroxide
LA	=	linoleic acid
LDH	=	lactate dehydrogenase
LDL	=	low-density lipoprotein
LSD	=	least significant difference
Mac(F)	=	fermented *C. maculata*
Mac(U)	=	unfermented *C. maculata*
MAD	=	malonaldehyde
MeOH	=	methanol
NADH	=	β-nicotinamide adenine dinucleotide
NaOH	=	sodium hydroxide
NBT	=	4-nitroblue tetrazolium chloride
O_2	=	oxygen
$O_2^{\bullet-}$	=	superoxide anion radical
$OH^{\bullet}$	=	hydroxyl radical
PG	=	propyl gallate

PMS	=	phenazine methosulphate
PUFA	=	polyunsaturated fatty acids
RO	=	reverse osmosis
$RO^{\cdot}$	=	alkoxyl radical
$ROO^{\cdot}$	=	peroxyl radical
ROOH	=	linoleic acid hydroperoxides
ROS	=	reactive oxygen species
Ses(F)	=	fermented *C. sessiliflora*
Ses(U)	=	unfermented *C. sessiliflora*
SOD	=	superoxide dismutase
Sub(F)	=	fermented *C. subternata*
Sub(U)	=	unfermented *C. subternata*
TAA	=	total antioxidant activity
TBARS	=	thiobarbituric acid reactive substances
TEAC	=	Trolox equivalent antioxidant concentration
TOC	=	α-tocopherol
UD	=	unfermented diethyl ether fraction
UM	=	unfermented methanol fraction
UV	=	ultraviolet
VIS	=	visible
XA	=	xanthine
XOD	=	xanthine oxidase

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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

Honeybush, representing more than 20 species from the genus *Cyclopia*, belongs to the indigenous legume family (Fabaceae) of the fynbos region in the Western and Eastern Cape Provinces, South Africa (Van Wyk *et al.*, 1997). In the past century honeybush tea was only consumed locally as a traditional herbal beverage. It was prepared by brewing the fermented leaves and stems of several *Cyclopia* species to obtain a pleasant-tasting drink with a "honey-like" flavour (Du Toit *et al.*, 1998). Currently, it is mainly *C. intermedia* which is harvested from both natural populations and experimental plantations for processing. This exclusive South African herbal tea industry is still relatively small, but has progressed to such an extent over the past few years that honeybush tea (mainly *C. intermedia*) is available in local supermarkets. Only about four tons of honeybush tea was exported in 1993 (Anonymous, 1996). During the 1999/2000 season *ca.* 100 tons were produced of which *ca.* 80% was exported, compared to the *ca.* 50 tons produced in the 1998/99 season. Germany is currently the largest market and smaller quantities are exported to the United Kingdom and the United States of America, while other potential export markets include Japan and the Netherlands (N. Coetzee, Coetzee & Coetzee Distributers, Cape Town, South Africa, personal communication, 2000).

Honeybush tea, apart from being enjoyed as a herbal beverage, has also been associated with certain health claims based on anecdotal evidence. These include the treatment of arthritis and certain skin ailments (such as psoriasis), as well as providing relief from urinary and digestive problems (Du Toit *et al.*, 1998). Compared to black tea (*Camellia sinensis*), honeybush tea contains no or traces of caffeine and has a very low tannin content (Terblanche, 1982; Van Wyk *et al.*, 1997). The increasing demand for honeybush tea, both locally and internationally, complies with the world-wide trend of an increased consumption of herbal infusions (Hasler, 1998). The total consumption of herbal and fruit infusions for Germany alone increased from 35.1 to 50.7% between 1986 and 1996, thereby outnumbering the consumption of regular black teas which

declined from 56.2 to 43.5% within the same period (Beerbaum, 1997). According to the German Tea Council, this remarkable increase in popularity of herbal and fruit infusions originates from the consumer's perception of it being healthy, pure, natural and caffeine-free (Beerbaum, 1997). In 1993, the UK herbal drink market expanded by 35% resulting in a market value of £45 million, again as a result of the association of herbal and tea based drinks with a healthier lifestyle (Cave, 1995).

An essential step in understanding the potential health properties of honeybush tea was the characterization of its phenolic composition. De Nysschen *et al.* (1996) showed varying combinations of three major compounds, mangiferin, hesperetin and isosakuranetin, in methanol extracts prepared from the dried unfermented leaves of 22 different *Cyclopia* species. Analysis of the phenolic composition of fermented *C. intermedia* showed the presence of flavanones, flavones, isoflavones, coumestans, xanthones and a phenolic acid (Ferreira *et al.*, 1998). These classes of phenolic compounds are associated with a vast diversity of pharmacological properties, including anti-inflammatory, antimicrobial, antiviral (Middleton & Kandaswami, 1993) and phytoestrogenic activities (Miksicek, 1995), as well as the lysosomal membrane stabilization effect of the xanthone mangiferin (Beecher *et al.*, 1990). Previous research on these phenolic metabolites, including the flavanones eriodictyol, hesperetin, hesperidin and naringenin, the flavone luteolin, the xanthone mangiferin, and the phenolic acid 4-coumaric acid, showed antioxidant and antiradical activities in various *in vitro* and *in vivo* model systems (Sato *et al.*, 1992; Joyeux *et al.*, 1995; Haraguchi *et al.*, 1996; Rice-Evans *et al.*, 1996; Miyake *et al.*, 1997).

The consumption of a diet rich in phytochemicals, i.e. plant-derived substances that are nutritionally, physiologically and/or medicinally highly active (Lin, 1994), including flavonoids, supplies the human body with exogenous antioxidants. These are essential in replenishing the endogenous antioxidant defence against oxidative damage (Ramarathnam *et al.*, 1995; Swanson, 1998). Within the last decade, accumulating evidence has repeatedly emphasized the important relationship between food consumption and the prevention and treatment of diseases, such as cancer, cardiovascular disease and hypertension (Bloch & Thomson, 1997; Goldberg, 1994). Worldwide, the magnitude of the consumer's demand for health-enhancing foods has contributed to an explosion in the functional foods market (Hasler, 1998). According to

Giese & Katz (1997), the total functional food market reached $171.1 million in 1997 and is expected to reach $314.1 million in 2002. Decision Resources, however, estimated the market value of functional foods for 1998 at $28.9 billion (Witwer, 1998).

The protective properties of naturally occurring plant compounds are, however, not solely reserved for the enhancement of human health, but also find broad application in the food and cosmetic industries. One of the major causes of food quality deterioration during processing, handling, transport and storage is lipid oxidation (Shahidi & Naczk, 1995). Suppression of these deleterious chains of reactions, initiated by free radical attack, is not only extremely important to preserve the nutritional value and sensory qualities of food, but also to ensure that food is non-toxic for consumption. The most effective way of delaying or preventing this oxidation process of lipid-containing materials is by the addition of antioxidants (Shahidi & Naczk, 1995). However, synthetic antioxidants, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), which are widely used in food (Shahidi & Naczk, 1995) and cosmetic industries (Pettigrew, 1997) to retard oxidation, have recently been shown to exert toxic side effects *in vivo* (Aruoma *et al.*, 1990). The urgent desire for safer alternatives to synthetic antioxidants prompted the investigation of the antioxidant potential of naturally-occurring compounds by researchers worldwide.

The potential of honeybush tea becoming a major industry in the competitive herbal tea and antioxidant-rich beverage markets has, therefore, initiated this investigation of its antioxidant and radical scavenging activities. Several species are currently being considered for large scale cultivation (Coetsee, 1999), and thus their antioxidant potential needs to be evaluated, especially as interest in marketing of honeybush for a functional food/nutraceutical market is increasing. Seasonal variation, harvesting practices, processing, prolonged storage and preparation methods may unavoidably change the phenolic composition of food with a potential impact on its antioxidant status (Piccaglia *et al.*, 1997; Lindley, 1998; Manzocco *et al.*, 1998; Ewald *et al.*, 1999).

The influence of fermentation on the antioxidant and antiradical properties of two commercially important species, *C. intermedia* and *C. subternata*, and three species showing potential commercial importance, *C. genistoides*, *C. maculata* and *C. sessiliflora* were, therefore, investigated in the study described here. For this

purpose, the superoxide anion radical ($O_2^{\bullet-}$) scavenging ability (Robak & Gryglewski, 1988), hydrogen-donating ability towards the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) (Brand-Williams *et al.*, 1995), and inhibitory effect towards linoleic acid peroxidation (Lingnert *et al.*, 1979), were quantified. Crude methanol and diethyl ether polyphenol-containing fractions, prepared by selective extraction of plant material from fermented and unfermented *C. intermedia*, were tested using the same methods. Several of the phenolic compounds of fermented *C. intermedia*, were also tested to determine their relative radical-scavenging efficiencies. Furthermore, the effects of extraction time and the presence of woody material in the processed product on the scavenging ability of fermented *C. maculata* (*ex* Du Toitskloof) towards DPPH$^{\bullet}$ and $O_2^{\bullet-}$ were investigated. This work was conducted in an effort to select the best preparation method that ensures maximum release of antioxidants. Quercetin and the synthetic antioxidants, BHA and BHT, were tested as reference compounds in the DPPH$^{\bullet}$ scavenging method. For the $O_2^{\bullet-}$ scavenging method, these compounds and an antioxidant enzyme superoxide dismutase (SOD), were utilised as reference compounds.

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Chapter 2

Literature Review

All substances are poisons... the right dose differentiates a poison from a remedy.
Paracelsus, 15th century

Plant foods, including fruit, legumes, grains, teas, herbs and spices, contain phenolic metabolites, some of which are associated with potent antioxidant activity (Shahidi & Wanasundara, 1992). Consumption of a diet rich in plant-derived substances could protect against oxidative damage (Ramarathnam *et al.*, 1995). In this literature review, the consequences of oxidative stress on antioxidant defence in food systems and the human body, and the concept of functional foods (eg. tea) are discussed briefly. Furthermore, honeybush tea and its polyphenol profile, beneficial properties associated with a polyphenol-rich diet, structure-activity relationship of polyphenols, and methods to assess antioxidant activity are considered.

The 'Oxygen Paradox'

Molecular oxygen present in the atmosphere as a stable triplet bi-radical in its ground state ($^3O_2$) (Green & Hill, 1984) is vital for existence of eukaryotic organisms. During normal cell metabolism O_2 is enzymatically reduced via a concerted four-electron (tetravalent) process by the mitochondrial electron transport chain to produce water (H_2O). In contrast to this relatively safe process, the spontaneous non-enzymic univalent reduction of O_2 in the cellular milieu produces reactive oxygen species (ROS), including superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^{\bullet}$) radicals (Fig. 1) (Davies, 1995). Oxidative stress, the inescapable consequence of living in an aerobic environment, refers to an imbalance within a biological system between O_2-derived species and antioxidant defence (Davies, 1995).

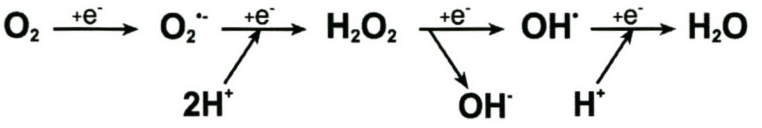


Figure 1 The spontaneous non-enzymic univalent reduction of O_2 in the cellular milieu produces reactive oxygen species (ROS), including superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^{\bullet}$) radicals (Davies, 1995).

Superoxide anion radicals ($O_2^{\bullet-}$) are not very reactive in an aqueous environment but may cause extensive peroxidative damage when generated within the hydrophobic interior of biological membranes (Gutteridge & Halliwell, 1994). The superoxide anion radical ($O_2^{\bullet-}$) is converted to $OH^{\bullet}$, the most reactive O_2 radical, merely by the addition of a single proton to this mild reductant (Davies, 1995). In addition, excessive $O_2^{\bullet-}$ reacting with H_2O_2 in the presence of iron and copper ions catalyses the formation of $OH^{\bullet}$ (Gutteridge & Halliwell, 1994). This highly reactive radical attacks all biologically important molecules including proteins, lipids, carbohydrates and nucleic acids (DNA, RNA) leading to cell and tissue injury (Davies, 1995; Gutteridge & Halliwell, 1994). The occurrence of mutations in genetic material, which form a fundamental part of the cancer process (Bender, 1998), is for instance due to $OH^{\bullet}$ reacting with constituents of DNA, eg. guanine (Fig. 2) (Gutteridge & Halliwell, 1994).

A possibility exists that formed radicals are involved in the ageing process of tissues (Gutteridge & Halliwell, 1994). Other implications of oxidative stress include numerous degenerative diseases and syndromes, including mutagenesis and cancer, arteriosclerosis, cardiovascular diseases (eg. coronary heart disease), chronic inflammatory diseases (eg. rheumatoid arthritis), acute inflammatory problems (eg. wound-healing), photo-oxidative stress to the eye (eg. cataracts) and central nervous-system disorders (eg. Parkinson's and Alzheimer's diseases) (Davies, 1995).

Lipid peroxidation

Oil easily combines with oxygen. This combination is either slow or rapid.
In the first case rancidity is the consequence, in the second inflammation.
Chaptal (1791)

Oxidative attack of membrane phospholipids, resulting in peroxidation of poly-unsaturated fatty acids (PUFAs), is an oxidative event that occurs most frequently within the human body (Buettner, 1993). It is thought to be one of the principle mechanisms that evoke cellular damage (Younes & Siegers, 1981) with a substantial role in some human diseases (Halliwell & Chirico, 1993). Furthermore, lipid peroxidation is involved in food quality deterioration during storage and processing (Bondet *et al.*, 1997).

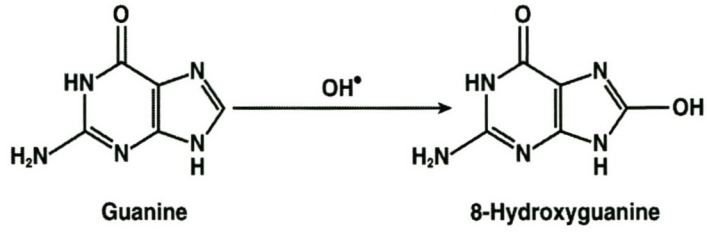


Figure 2 The reaction of $OH^{\bullet}$ with constituents of DNA , eg. guanine, results in the occurrence of mutations in genetic material.

The classical route of lipid peroxidation involving complex free-radical-mediated chain reaction processes is summarised in the following steps (Shahidi & Naczk, 1995):

(i) ***induction*** or ***initiation***, during which lipid free radicals ($R^{\bullet}$) and a hydrogen atom (H^{+}) is formed:

$$RH + \text{initiator} \rightarrow R^{\bullet} + H^{+}$$

(ii) ***propagation***, during which these highly reactive chemical species produce lipid hydroperoxides (ROOH) via peroxyl radicals ($ROO^{\bullet}$):

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$
$$ROO^{\bullet} + RH \rightarrow R^{\bullet} + ROOH$$

(iii) ***termination***, during which two radicals are associated to form stable non-radical products:

$$R^{\bullet} + R^{\bullet} \rightarrow \text{non-radical product}$$
$$R^{\bullet} + ROO^{\bullet} \rightarrow \text{non-radical product}$$
$$ROO^{\bullet} + ROO^{\bullet} \rightarrow \text{non-radical product}$$

Food quality deterioration

Oxidative deterioration of polyunsaturated lipids in food is generally initiated either by exposure to light, heat, ionizing radiation, metal ions, metalloprotein catalysts or by the enzyme lipoxygenase (Shahidi & Naczk, 1995). The result may either be nutritional losses (i.e. vitamin destruction), discoloration (Yen & Duh, 1994), the formation of off-flavours (Eiserich *et al.*, 1995), textural changes (Shahidi & Naczk, 1995), the disappearance of essential fatty acids (Bondet *et al.*, 1997), or formation of undesirable chemical compounds which may be detrimental to health (Brand-Williams *et al.*, 1995).

Cellular damage and disease

The abundance of PUFAs in the hydrophobic interior of biological membranes and their presence in an O_2-rich, metal-containing environment, make them prone to peroxidative attack by free radicals resulting in lipid peroxidation (Fig. 3) (Buege & Aust, 1978). A controversy surrounds $OH^{\bullet}$: it is often proposed as the main initiator of membrane lipid peroxidation, whereas some researchers have shown that $OH^{\bullet}$ scavengers did not inhibit lipid peroxidation. The short-lived nature of $OH^{\bullet}$ also makes it difficult to migrate to the hydrophobic interior of membranes (Minotti & Aust, 1987). Gutteridge & Halliwell (1994) proposed that $OH^{\bullet}$ can initiate lipid peroxidation by abstracting H^{+}, which is covalently bound to carbon in the fatty acid backbone of a PUFA side chain:

$$\mathbf{C\text{-}H + OH^{\bullet} \rightarrow C^{\bullet} + H_2O}$$

Reaction of the carbon-centred radical ($C^{\bullet}$) with O_2 in the hydrophobic interior of membranes results in the production of highly reactive $ROO^{\bullet}$, the major chain-propagating step in lipid peroxidation (Gutteridge & Halliwell, 1994).

Increasing evidence shows the association between oxidative damage to low density lipoprotein (LDL) cholesterol, an essential lipid to maintain the phospholipid bilayer structure in biological membranes, and the risk of coronary heart disease (CHD) (Gutteridge & Halliwell, 1994; Tijburg *et al.*, 1997). Apart from damage to membrane lipids, peroxidation also results in damage to proteins and depletion of antioxidants (Gutteridge & Halliwell, 1994).

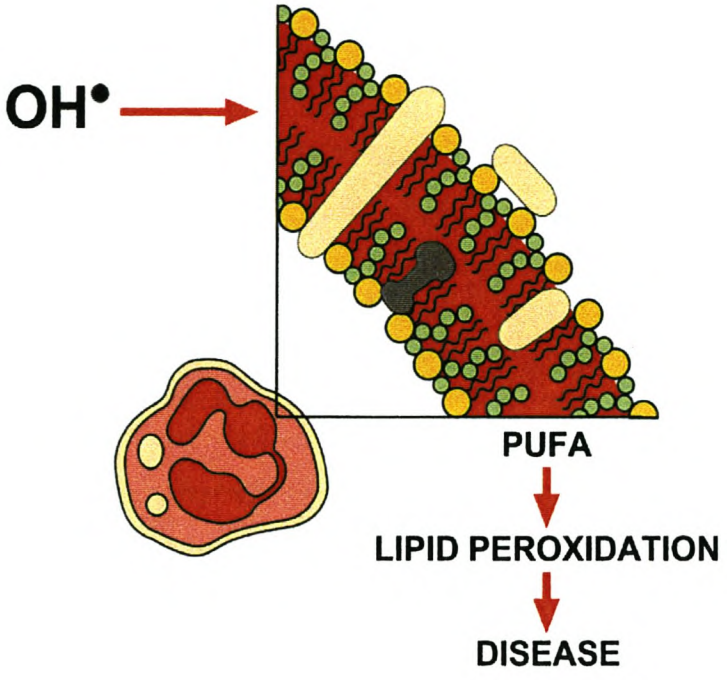


Figure 3 The abundance of polyunsaturated fatty acids (PUFAs) in the hydrophobic interior of biological membranes make them prone to peroxidative attack by free radicals (eg. $OH^{\bullet}$), resulting in lipid peroxidation (Buege & Aust, 1978), damage to proteins, depletion of antioxidants and probably disease (Gutteridge & Halliwell, 1994).

Antioxidants

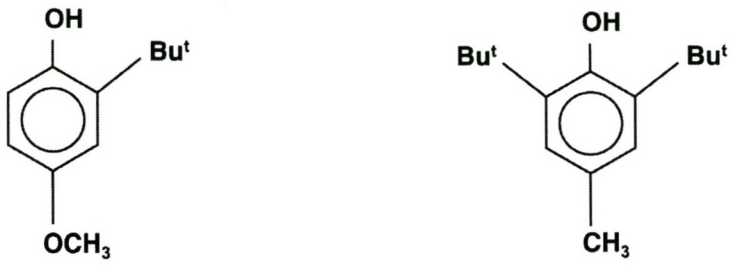
"antioxidants are substances used to preserve food by retarding deterioration, rancidity or discoloration due to oxidation"
USDA Code of Federal Regulations 170.3 (0) (3)
Dziezak, 1986

An antioxidant is any substance that, when present at low concentrations compared with those of an oxidizable substrate (including proteins, lipids, carbohydrates and DNA), significantly delays or prevents oxidation of that substrate.
Halliwell & Gutteridge, 1989

An antioxidant may protect a target molecule by scavenging O_2-derived species, chelating catalytic metal ions, repairing damage to the target, or by substituting damaged target molecules with new ones (Gutteridge & Halliwell, 1994; Shahidi & Naczk, 1995). Some antioxidants react with high energy lipid radicals thereby converting them to thermodinamically more stable products, while other antioxidants function by retarding the rate of chain initiation by breaking down hydroperoxides. It is essential to protect food lipids and human tissues against free radicals by endogenous and exogenous antioxidants from a natural or synthetic origin (Bondet *et al.*, 1997).

Food antioxidants

The addition of antioxidants to lipid-containing food has been shown to be effective in retarding the oxidation of fats (Brand-Williams *et al.*, 1995; Yen & Duh, 1994), thereby extending the shelf-life of foodstuffs and reducing wastage and nutritional losses (Shahidi & Naczk, 1995). An ideal food-grade antioxidant is safe, does not contribute to colour, odour or flavour, is effective at low concentrations, is easy to incorporate, survives after processing, is stable in the finished product and is available at low cost (Coppen, 1983). Synthetic antioxidants, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) (Fig. 4) and propyl gallate (PG), are widely used in the food (Duh, 1998) and cosmetic industries (Pettigrew, 1997) to retard oxidation.



BHA **BHT**

Figure 4 Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are synthetic antioxidants which are widely used in the food (Duh, 1998) and cosmetic industries (Pettigrew, 1997) to retard oxidation.

The possible toxicity of synthetic chemicals *in vivo* has caused an increasing awareness among consumers about the safety of food additives (Aruoma *et al.*, 1990; Kim *et al.*, 1994; Yen & Duh, 1994). Symptoms include an enlarged liver and increased microsomal enzyme activity, while conversion of some material to toxic or carcinogenic substances also takes place (Farag *et al.*, 1989). This prompted the investigation of the antioxidant potential of naturally occurring compounds as safer alternatives to synthetic food additives (Basaga *et al.*, 1997; Farag *et al.*, 1989; Yen & Duh, 1994) and explains the increasing consumer preference of natural products (Bondet *et al.*, 1997). Flavonoids, the most potent antioxidative compounds of plant phenolics, need further investigation in order to explore the feasibility of their use in foods and to determine their toxicological implications (Shahidi & Naczk, 1995).

In vivo antioxidant protection

Most living organisms possess extremely efficient protective systems against the damaging effects of ROS. In the human body for instance, several antioxidant defence systems exist including the superoxide dismutase (SOD), glutathione peroxidase and catalase enzymes, and antioxidant vitamins such as ascorbic acid and vitamin E (Gutteridge & Halliwell, 1994; Halliwell, 1995). These compounds may prevent the damaging effects of oxidative stress on target molecules by interrupting the free radical pathway of lipid peroxidation (Meyer *et al.*, 1994; Summanen *et al.*, 1995), thereby prolonging the oxidation-induction period (Yan & White, 1990). Superoxide dismutase (SOD) catalyses the disproportionation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (McCord & Fridovich, 1969), while catalase is further responsible for the conversion of H_2O_2 to H_2O and O_2 (Davies, 1995).

The capability of these protective systems may, however, gradually decrease with age, resulting in an imbalance in the normal redox equilibrium which exists in healthy systems (Ramarathnam *et al.*, 1995). This will subsequently lead to malfunctioning of vital organs, an increased susceptibility to diseases readily associated with ageing, and eventually death (Ramarathnam *et al.*, 1995). The endogenous antioxidants distributed in and around living cells, which regulate the various oxidation-reduction reactions, are therefore seen as a potential class of determinants of longevity. To replenish the age-

induced loss in the capability of endogenous antioxidant defence mechanisms against oxidative damage, it is essential to consume a diet rich in plant-derived substances that are nutritionally, physiologically and/or medicinally highly active, the so-called phytochemicals (Lin, 1994). These include flavonoids, ascorbic acid, α-tocopherol and the carotenoids (Ramarathnam *et al.*, 1995; Swanson, 1998). Epidemiological studies, *in vitro* and *in vivo* studies, as well as clinical trial data indicate that a plant-based diet can reduce the risk of chronic disease, such as cancer (Hasler, 1998).

The family of phenolic compounds

Plant polyphenols, for example tannins and flavonoids, are secondary metabolites which are widely distributed in the higher plant kingdom (Haslam, 1996). All phenolics, including phenolic acids, coumarins, lignans, lignins and flavonoids, are partially derived from phenylalanine (Bi *et al.*, 1997). Flavonoids, for instance, contain two benzene rings in the basic structure, one of which arises from phenylalanine, but the other benzene ring is derived from acetyl-CoA via the polyketide pathway (Bi *et al.*, 1997; Hollman *et al.*, 1996). A diversity of over 4 000 naturally occurring flavonoids exist through variation in their heterocyclic rings, giving rise to monomeric flavanols (*i.e.* catechins, leucoanthocyanidins), biflavonoids, biflavans, flavonols, flavones, flavanones, anthocyanidins, proanthocyanidins, isoflavonoids and chalcones (Bors *et al.*, 1990; Hertog, 1996; Hollman *et al.*, 1996; Shahidi & Naczk, 1995).

The phenolic structures of flavonoids, of which the most common ones include anthocyanins, flavonols and flavones, suggest that they may possess antioxidative properties. Flavonoids generally occur as *O*-glycosides with sugars bound at the C3 position (Hertog *et al.*, 1993). Flavonols and flavones only occur as glycosides in edible parts of plant foods, i.e. with a sugar moiety bound to various positions in the ring of the parent flavonoid (Hollman *et al.*, 1996). The sugar-free part of the flavonoid molecule is the aglycone (Hertog, 1996).

Consumption of flavonoids

According to Kühnau (1976), important dietary sources of flavonoids include fruits,

vegetables and beverages, of which the latter represent approximately 25 - 30% of the total daily flavonoid intake. A study of foods commonly consumed in The Netherlands, showed that black tea, a rich source of glycosides (Finger *et al.*, 1991), contributes to 48% of the total intake of flavonoids, compared to 29% by onions and 7% by apples (Hertog, 1996). Hertog (1996) determined that the average intake of flavonols and flavones are approximately 23 mg per day, of which the flavonol quercetin (Fig. 5) is the most predominant at 16 mg per day.

This data contradicts the estimation of Kühnau (1976) that the average Western diet contains approximately 1 g of mixed flavonoids (expressed as glycosides), which is equivalent to approximately 115 mg of flavonol and flavone aglycones per day. The flavonoid content used by Kühnau (1976) was probably overestimated by using incorrect methods and by including the flavonoid content of non-edible parts of plants (Hollman *et al.*, 1996). The average intake of isoflavones are approximately 25 - 100 mg per day for Asians who consume the equivalent of 10 - 35 g of soybeans per day (Coward *et al.*, 1993). It is, however, unlikely that the average US or Western European consumer ingests more than a few mg of isoflavones per day (Coward *et al.*, 1993; Kardinaal *et al.*, 1997).

Functional foods (Nutraceuticals)

Let thy food be thy medicine and thy medicine be thy food.
Hippocrates

Functional foods differ from conventional foods in that it contains significant levels of biologically active components thereby offering a possible health benefit by means of disease prevention and increased body functioning (Goldberg, 1994; Hasler, 1998). Contributory factors to functional food development include, an ageing population, the trend towards self-medication, rising healthcare costs, scientific breakthroughs and a growing awareness among consumers regarding diet and health. On the other hand, slow legislation developments, conflicting and inadequate scientific evidence, consumer suspicion and confusing messages, are factors which impede functional food growth (Crates, 2000).

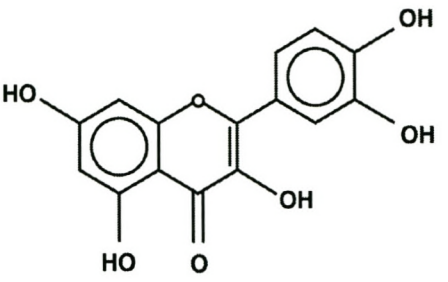


Figure 5 The chemical structure of the well-known food antioxidant quercetin.

The functional food concept is in fact attributed to the Japanese who, already in 1935, developed the world's current leading functional food product, Yakult (a lactic acid-enriched dairy product) (Young, 1996). However, only in the mid-1980's, the term "functional foods" was introduced in Japan (Hasler, 1998). This is, to date, the only country with a specific regulatory approval process which evaluates the potential health claims for functional foods, i.e. the "Foods for Specified Health Use" (FOSHU) system that originated in 1991 (Burke, 1997). FOSHU approval indicates that a food was scientifically evaluated, that its medicinal and/or nutritional claims have a clear basis and that the recommended dose is established (Burke, 1997). The Japanese Ministry of Health and Welfare included phytochemicals and antioxidants in their list of food ingredients with health-enhancing properties in an effort to improve their national diet (Goldberg, 1994). By 1998, about 100 food products were licensed as FOSHU foods in Japan, thus bearing the seal of approval of the Japanese Ministry of Health and Welfare (Hasler, 1998). Some examples of such foods include lactic acid bacterial culture drinks (eg. Yakult, Calpis and Lactia), soy protein-containing meat and tofu products, and calcium-enriched soft drinks (Burke, 1997).

Functional foods (mainly dairy products) were also introduced in the European markets in the mid-1980's. Although the European claims legislation is very restrictive, a wide selection of functional foods were already available on the European market by 1996. Such developments include mainly already existing foods that were developed to improve health, eg. probiotics in yoghurt, soluble fibre added to fruit juice or yoghurt, omega-3-enriched spreads, folic acid-containing milk and calcium-enriched products (Young, 1996). In the USA, the term "functional food" was until 1998 not legally recognised (Hasler, 1998), but is currently more commonly used by the industry (Witwer, 1999). The term "antioxidant" may only be used to describe a nutrient when scientific evidence shows that absorption of sufficient quantities of the nutrient result in either inactivation of free-radicals or prevention of free radical-initiated chemical reactions in the human body (Giese & Katz, 1997). Health claims about the use of a dietary supplement or food to prevent, treat or cure a specific disease, are illegal in the USA, unless such claims are authorized by the Food and Drug Administration (FDA) (Giese & Katz, 1997; Witwer, 1999). Since the Nutrition Labelling and Education (NLEA) Act of 1990, which grants the right to companies to make a health-medical claim

on a specific food or category of foods (DeFelice, 1998), only 11 health claims gained FDA approval (Witwer, 1999). Two of these claims are, for instance, the beneficial relationships between calcium and osteoporosis, and between soluble fibre and heart disease (Witwer, 1999). The Dietary Supplement Health and Education Act (DSHEA), unlike NLEA, permits companies to make claims on dietary supplements, regarding structure-function, mechanism of action, well-being and classic deficiencies, without prior FDA approval. This law prohibits claims regarding the diagnosis, treatment, litigation, cure and prevention of disease (DeFelice, 1998).

In South Africa, a framework for the labelling of health claims already exists for certain foods and ingredients. Any health claim should be based on sound scientific evidence before the regulatory authorities allow such claims on a food product (Reid, 1997).

Dietary fibre, oligosaccharides, vitamins, probiotics, isoflavonoids and PUFAs are all marketed under the functional food/nutraceutical banner (Hasler, 1998; Witwer, 1999). The difficulty in defining and categorising functional foods, therefore, results in significant contradictions in estimations of the functional food market size (Hasler, 1998; Crates, 2000). The total market value for functional foods reached $171.1 million in 1997, but it is expected to reach $314.1 million in 2002 due to the increased consumer awareness and interest in health-enhancing foods (Giese & Katz, 1997). The European functional food market alone was estimated at $4 - 8 billion, $5 billion and $6.6 billion by the respective market research firms, Foodlink Forum, PA Consulting and Datamonitor, Inc. (Crates, 2000). The latter company furthermore reported that the US nutraceutical market alone has grown from $11 to $16.7 billion from 1994 to 1998 (Pszczola, 1998), whereas Decision Resources estimated the market value of functional foods at $28.9 billion in 1998 (Witwer, 1998). However, according to the Foundation for Innovation in Medicine (FIM), the magnitude of the US nutraceutical market was estimated at $250 billion, which is *ca.* 2.5 times larger than the US pharmaceutical market. This figure was based on 1992 data of the American diet and the assumption that at least 50% of food is consumed for medical or health reasons (DeFelice, 1998).

Tea as a functional beverage

The rich history of tea (*Camellia sinensis*) starts in Ancient China and dates as far back as 2 737 B.C., when leaves from a wild tea plant accidentally fell into a bowl of hot water resulting in an infusion enjoyed by the mythological emperor Shen Nung (Balentine, 1997). Tea was introduced to Europe at the beginning of the 17th century, when China was the world's sole tea producer, and soon thereafter tea drinking became popular in Britain (Balentine *et al.*, 1997). Tea-drinking which was regarded as a symbol of hospitality was mainly a social affair associated with elite circles. However, tea is currently consumed daily as a refreshing beverage and has also gained popularity as a health drink (Knox & Lowman, 1997). In 1996, the worldwide tea production totalled 2 610 569 metric tons thereby showing its major commercial importance (Balentine *et al.*, 1997).

Fresh green leaves, green (unfermented) and black (fermented) teas contain approximately 30 - 35, 10 - 25 and 8 - 21% tea polyphenols, respectively. The major green tea catechin, epigallocatechingallate (EGCG), is possibly responsible for the antioxidant activity against oxidation of fat induced in the Rancimat test (Lunder, 1992). Ruch *et al.* (1989) reported that green tea antioxidant (GTA), i.e. catechins, showed antioxidant activity towards H_2O_2 and $O_2^{\bullet-}$. In the process, GTA prevents H_2O_2- and $O_2^{\bullet-}$-induced cytotoxicity and inhibition of intercellular communication in cell culture. Evidence obtained from *in vitro* and *in vivo* studies using experimental animals suggests that green and black tea extracts at concentrations usually consumed by humans, and tea polyphenols, may inhibit the carcinogenic process (Dreosti *et al.*, 1997). Evidence obtained from several epidemiological studies indicating a relationship between tea consumption (especially green tea) and reduced cancer risk among humans, has still to be confirmed (Dreosti *et al.*, 1997). Eight cohort studies reporting on the relationship between black tea consumption and cancer risk showed contradictory results (Blot *et al.*, 1997). These varying results may be ascribed to differences in the bioavailabilities and activities of relevant active tea components in the body. Knowledge of absorption, metabolism, tissue distribution and biological activities of active components is thus essential to comprehend and interpret epidemiological evidence of tea consumption (Hollman *et al.*, 1997).

Honeybush in general

Honeybush is an indigenous legume plant, represented by *ca.* 24 species from the genus *Cyclopia*, belonging to the fynbos region in the Western Cape, South Africa (Van Wyk *et al.*, 1997). *Cyclopia genistoides* mainly grows in the west and along the Southern Cape Coast, whilst *C. intermedia* ("bergtee") and *C. subternata* ("vleitee") occur south and eastwards (Van Wyk *et al.*, 1997). In 1705, the honeybush plant was mentioned for the first time (Kies, 1951), but not necessarily for the purpose of consumption as an infusion. Over the past centuries, honeybush tea manufacturing has increased in popularity and became familiar among local inhabitants for use on a relatively small scale (Du Toit, 1996). The market demand for a consistently good quality product initiated the development of a standardised processing method (Du Toit & Joubert, 1998). This process involves fermentation of small pieces of leaves and stems under controlled conditions and subsequently drying of the fermented material to obtain a final product uniformly dark-brown in colour and size with a distinguished sweet "honey"-like flavour and aroma (Du Toit *et al.*, 1998). Presently, honeybush tea is sold locally and exported to several countries, but the industry is still relatively small. Only *ca.* 20 tons of honeybush tea (*C. intermedia*), of which most was for export, were produced annually between 1994 and 1996 by the three major tea producers in the Langkloof area of the Southern Cape (Du Toit, 1996). Honeybush tea production (mainly *C. intermedia*) has, however, increased to *ca.* 100 tons in the 1999/2000 season of which *ca.* 80% was exported, thereby showing a 100% increase in production from the previous season (N. Coetzee, Coetzee & Coetzee Distributers, Cape Town, South Africa, personal communication, 2000). *Cyclopia intermedia*, which has major commercial importance, is the only species for which the phenolic profile has been characterised (Ferreira *et al.*, 1998), whilst that of *C. subternata* is currently under investigation. The phenolic profiles of other species also needs to be investigated in order to enhance the utilization of honeybush tea and for the continued growth of the industry in a competitive herbal tea market, both locally and internationally.

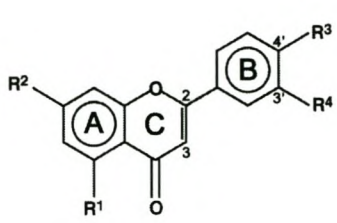
Phenolic compounds in honeybush tea

A variety of $C_6.C_3.C_6$-type polyphenols (flavanones, isoflavones, flavones and flavonols), coumestans, xanthone C-glycosides, a hydroxy-cinnamic acid, and $C_6.C_1$- and $C_6.C_2$-type phenols, were isolated from fermented *C. intermedia* (Ferreira *et al.*, 1998; Kamara, 1999). Only those compounds that were selected for testing of their antioxidant and antiradical potential in the present study are illustrated in Fig. 6. It was cited by Ferreira *et al.* (1998) that some of these phenolic compounds are associated with a vast diversity of pharmacological properties, which may explain some of the anecdotal health claims associated with honeybush tea (Du Toit *et al.*, 1998).

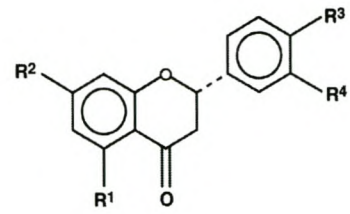
The efficiency of a honeybush infusion to relieve arthritis (G. Kritzinger, Misgund, South Africa, personal communication, 1997) is probably due to the presence of pseudobaptigen, an isoflavone present in fermented *C. intermedia* (Ferreira *et al.*, 1998), that is used for the treatment of arthritis. An inositol, (+)-pinitol, which was isolated from fermented *C. intermedia* (Ferreira *et al.*, 1998), is normally applied as an expectorant (Beecher *et al.*, 1989). This explains the use of a honeybush tea infusion as an expectorant in chronic catarrh and pulmonary tuberculosis (Bowie, 1830). Honeybush tea contains no caffein and has a very low tannin content. It is furthermore associated with the treatment of insomnia (sleeplessness), stomach or digestive problems and for curing certain skin ailments (eg. psoriasis) (Du Toit *et al.*, 1998). The functional food potential of this herbal beverage, therefore, probably lies therein that it could offer a possible health benefit to the consumer by enhancing the body's functioning and by preventing illnesses/diseases.

Impact of dietary intake of polyphenols

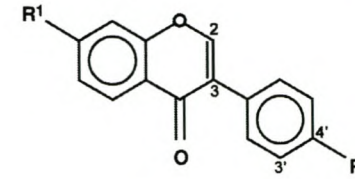
Dietary antioxidants may efficiently protect living systems from peroxidative damage, thereby playing an important role in prevention of carcinogenesis and in extending the life span of animals (Osawa *et al.*, 1990; Cutler, 1984). Cardiovascular disease risk is reduced by consumption of a diet rich in antioxidant flavonoids by preventing free radicals to oxidize LDL and by reducing platelet aggregation and thrombotic tendencies (Hertog, 1996).



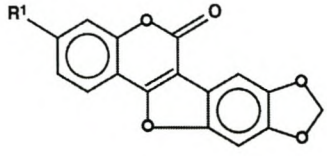
Flavone



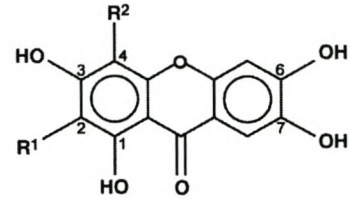
Flavanone



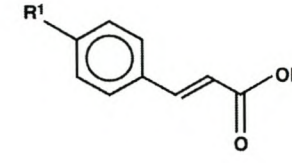
Isoflavone



Coumestan

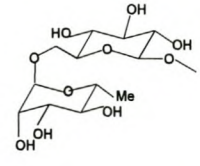


Xanthone



Phenolic acid

Group	Compound	R^1	R^2	R^3	R^4
Flavone	Luteolin	OH	OH	OH	OH
Flavanone	Eriodictyol	OH	OH	OH	OH
	Naringenin	OH	OH	OH	H
	Hesperetin	OH	OH	OCH_3	OH
	Hesperidin	OH	O-rutinosyl	OCH_3	OH
Isoflavone	Formononetin	OH	OCH_3		
Coumestan	Medicagol	OH			
	Flemichapparin	OCH_3			
Xanthone	Mangiferin	2-β-*D*-gluco-pyranosyl	H		
	Isomangiferin	H	2-β-*D*-gluco-pyranosyl		
Phenolic acid	4-Coumaric acid	OH			

O-rutinosyl ≡ 

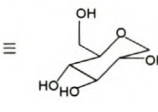
2-β-*D*-glucopyranosyl ≡ 

Figure 6 Structures of the polyphenolic compounds present in fermented *C. intermedia* (Ferreira *et al.*, 1998).

Flavonoids could also play an important role in lowering LDL peroxidation, thereby preventing atherosclerosis (De Whalley *et al.*, 1990). Apart from antioxidant properties, flavonoids may also exert other beneficial pharmacological effects including antiinflammatory and antiallergic effects (Middleton & Kandaswami, 1992) and maintaining healthy bones thereby protecting against osteoporosis (Brandi, 1992). Flavonoids (eg. quercetin, rutin, naringin and naringenin) may inhibit spontaneous *in vitro* lipid peroxidation resulting from a depletion of hepatic glutathione, the most prominent defence system which removes H_2O_2 and organic hydroperoxides from living cells (Younes & Siegers, 1981). Several flavonoids, including quercetin, luteolin and naringenin, suppress mutagenesis in *Salmonella typhimurium* strain TA100 induced by a direct-acting carcinogen (Huang & Ferraro, 1992).

In early studies it was shown by Rusznyàk & Szent-Györgyi (1936) that capillary permeability and fragility in humans were decreased by a mixture of two flavanones. Many flavonoids have potent antiradical activities, for example the most ubiquitous food antioxidant, quercetin, which scavenges $O_2^{\bullet-}$ (Robak & Gryglewski, 1988), $OH^{\bullet}$ (Husain *et al.*, 1987), and donates H^+ to $ROO^{\bullet}$ (Torel *et al.*, 1986). The strong biological activity of flavones is probably due to its highly methoxylated state (Benavente-García *et al.*, 1997).

Flavonols can function as antioxidants in biological systems by terminating radical chain reactions during lipid peroxidation and by quenching singlet molecular oxygen ($^1O_2$) which may initiate lipid peroxidation (Sorata *et al.*, 1984). Apart from antioxidative functions, flavonols have the ability to bind to biomembranes thereby protecting against vascular disorders (Sato *et al.*, 1992), for instance by decreasing the increasing permeability and fragility of capillaries (Sorata *et al.*, 1984). Other pharmacological properties of flavonols include anti-inflammatory and anti-allergic effects (Sato *et al.*, 1992).

Isoflavonoids, lignans, coumestans and resorcylic acid lactones are the major compounds of plant origin which possess oestrogenic activity, the so-called phyto-oestrogens (Verdeal & Ryan, 1979). Isoflavones, the most common naturally-occurring isoflavonoids, which are structurally related to flavones, have shown several beneficial health effects in humans, including the potential prevention of breast, prostate and colon cancers (Messina & Barnes, 1991), lowering of total and LDL cholesterol in blood

(Kardinaal *et al.*, 1997), thereby lowering the risk of cardiovascular disease (Anderson *et al.*, 1995), and the improvement of bone health by preventing postmenopausal bone loss (Bahram *et al.*, 1996; Kardinaal *et al.*, 1997). The isoflavones are also known to inhibit microsomal lipid peroxidation (Jha *et al.*, 1985). Some other effects which were reported for specific isoflavones include the inhibition of tyrosine-specific protein kinase, the inhibition of inositol phospholipid turnover (Imoto *et al.*, 1988) and antifungal activity (Weidenbörner *et al.*, 1989).

Apart from estrogenic activity, formononetin has also shown anticarcinogenic activities in sheep and rats and it inhibits the growth of human breast cancer cells (Chang *et al.*, 1994). It was reported that when comparing women consuming an isoflavonoid-rich diet with women with a low intake of such diets, it was found that the former have a lower risk of breast cancer (Chang *et al.*, 1994). According to Miksicek (1995), only the flavanones, 4',7-dihydroxyflavanone and 4',5,7-trihydroxyflavanone (naringenin) reproducibly activated the estrogen receptor compared to 3',5,7-trihydroxy-4'-methoxyflavanone (hesperetin), which was inactive. Structural features which are most important with respect to estrogenic activity include the diaryl ring structure which is common to all flavonoids and a minimum of one OH-substituent on each of these aromatic rings (Miksicek, 1995). The flavanone naringenin also showed significant antihepatotoxic effects (Hikino *et al.*, 1984).

Xanthones possess several biological properties including cytotoxic, antitumour, mutagenic, antimicrobial and anti-inflammatory activities (Rath *et al.*, 1996). These polyphenols also have an inhibitory effect towards monoamine oxidases (MAO) (Suzuki *et al.*, 1978; Rocha *et al.*, 1994) and it has possible potential as antidepressant drugs. Xanthone derivatives, which are widely distributed in higher plants, have pharmacological effects such as anti-allergic (Pfister *et al.*, 1972) and anti-platelet (Teng *et al.*, 1989) actions, as well as central nervous system stimulating and depressing effects (Sultanbawa, 1980). However, almost nothing is known about their antioxidant activity in biological systems (Sato *et al.*, 1992).

Specific mechanisms of antioxidant defence by polyphenols

Ratty & Das (1988) revealed structure-activity relationship for flavonoids by comparing

structures of different flavonoids with their potencies in ferrous sulphate-induced lipid peroxidation studies. They proposed that the presence of polyhydroxylated substitutions on A- and B-rings, a 2,3-double bond, a free 3-hydroxyl substitution and a 4-keto moiety, would confer potent antiperoxidative properties on the flavonoid molecule. Ratty & Das (1988) furthermore illustrated that the presence of a sugar moiety on the flavonoid molecule masked the antiperoxidative action of flavonoids; for example, naringenin, hesperetin and quercetin showed greater activity than their corresponding glycosides.

The recognition that the antioxidative and lipid-peroxidation inhibiting potential of flavonoids predominantly resides in their radical-scavenging capacity rather than the chelation of metals, evoked the problem of radical chemistry. As a result, three factors need to be considered when classifying a certain substance as a free radical terminating antioxidant:

Rate constants with different types of radicals

Bors *et al.* (1990) investigated the reactivity of commercially available hydroxylated and methoxylated flavonoid aglycones with selectively generated radicals by determination of reaction rate constants, which is dependant on O-H bound dissociation energy (Shahidi & Naczk, 1995). All radicals generated by pulse radiolysis in this investigation, including hydroxyl ($OH^{\bullet}$), azide ($N_3^{\bullet}$), superoxide ($O_2^{\bullet-}$), linoleic acid peroxyl ($LOO^{\bullet}$), *tert*-butoxyl (*t*-$BuO^{\bullet}$) and sulfite ($SO_3^{\bullet-}$) radicals, are oxidising species and it is assumed that, in reaction with phenolic compounds, aroxyl radicals will be formed (Bors *et al.*, 1990). Phenolic antioxidants act as chainblockers by donating H^+ to intermediate $ROO^{\bullet}$ (Kurechi *et al.*, 1980). Flavonoids react rapidly with $OH^{\bullet}$, considering the high reactivity of $OH^{\bullet}$ with aromatic compounds in general (Bors *et al.*, 1990). On the contrary, rate constants to scavenge $O_2^{\bullet-}$ are very low, even for kaempferol and quercetin, two of the most efficient radical scavengers (Bors *et al.*, 1990). The rate constant of hesperetin in reaction with $O_2^{\bullet-}$ is much lower than that of quercetin, indicating poor scavenging efficiency of the former phenol towards this particular radical (Jovanovic *et al.*, 1994). In general, an antioxidant should be an excellent donor of electrons or protons (Zhang & Chen, 1997).

According to Jovanovic *et al.* (1994), the reactivities of flavonoids with $O_2^{\bullet-}$

(i.e. reduction potential) depend on their reductive properties (i.e. electron-donating properties), which are highly sensitive to the substituents in the B-ring. An investigation of the reductive properties of model phenoxyl and selected flavonoid radicals showed that the antioxidant active site in any flavonoid is located in the ring having the lowest reduction potential (Rice-Evans, 1995; Jovanovic *et al.*, 1996);

Stability and decay kinetics of the produced antioxidant radical

Although most flavonoids are effective radical scavengers, this property by itself does not imply a beneficial effect because a flavonoid radical is formed after scavenging (Bors *et al.*, 1990). Therefore, the stability of the resultant free-radical intermediate or resonance delocalisation of the formed phenol radical (Shahidi & Naczk, 1995) is very important when determining the capability of a substance to act as an antioxidant (Zhang & Chen, 1997), considering that a very reactive secondary radical would propagate, rather than interrupt, the deleterious events initiated by a radical chain reaction. However, a flavonoid radical with high stability will not readily react, but will act as an antioxidant (Bors *et al.*, 1990); and

Stoichiometry of the radical-scavenging process

Effective antioxidants react in a 1:2 stoichiometry, i.e. one antioxidant molecule reacts with two radical species. The second reaction is a radical-radical recombination process, as been observed for aliphatic ROO$^{\bullet}$ reacting with phenolic and arylamine antioxidants and with α-tocopherol (Bors *et al.*, 1990).

The presently available evidence on the formation and decay of flavonoid aroxyl radicals served as a basis for the determination of the three structural features essential for radical scavenging and/or antioxidative potential and thereby confirms the research performed by Ratty & Das (1988). The criteria for effective radical scavenging by polyphenols thus include: (i) the 3',4'-dihydroxy (catechol) configuration in the B-ring which confers greater stability to aroxyl radicals, possibly through H-bonding, and which participates in electron delocalization; (ii) the 2,3-double bond in conjugation with the carbonyl group at position 4 (4-oxo function) in the C-ring, which is responsible for electron delocalization from the B-ring; and (iii) the additional presence of both 3- and

5-OH groups for maximum radical-scavenging potential and strongest radical absorption (Fig. 7). From a kinetic viewpoint, the 3- and 5-OH groups are equivalent owing to their H bonds with the 4-keto group (Bors *et al.*, 1990).

The antiradical capacity or reducing properties of any polyphenolic compound is directly influenced by the number and structural arrangement of the free and sterified phenolic hydroxyl groups (Benavente-García *et al.*, 1997; Rice-Evans, 1999). Roginsky *et al.* (1996) concluded from research on fifteen antioxidants, including quercetin, luteolin, naringenin and BHT, that the primary active sites of flavonoids are the phenolic hydroxyls of the B-ring. The flavones (Husain *et al.*, 1989), having two hydroxyl groups at positions 3 and 4 of the B-ring, and the isoflavones (Jha *et al.*, 1985), having the same groups at 6 and 7 of the A-ring, exhibited strong antioxidant potency.

The presence or absence of the 5-OH group may have a decisive influence on the configuration of the flavonoid, thereby affecting the electron delocalisation capacity of the compound and thus the stability of the flavonoid aroxyl radicals (Benavente-García *et al.*, 1997). Other structural features which may influence the mechanism of action of flavonoids, include the presence or absence of glycosidic moieties in the flavonoid skeleton and the site of glycosylation (Benavente-García *et al.*, 1997). The reactivity of the flavonoid structure is affected by steric hindrance derived from bulky groups, such as C- and O-glycosylated sugar moieties, substituting H in the aromatic ring structure (Shahidi & Naczk, 1995).

Green tea catechins possess effective structural features to scavenge 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) (Nanjo *et al.*, 1996) and $O_2^{\bullet-}$ (Hatano *et al.*, 1989), as well as 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfic acid radical cation ($ABTS^{\bullet+}$) in the aqueous phase (Salah *et al.*, 1995). These structural features include (i) the galloyl moiety attached at position 3 of the flavan-3-*ol* skeleton, and (ii) the *ortho*-trihydroxyl group in the B-ring. However, the presence of an *ortho*-dihydroxyl group in the B ring, is basically important for achieving a high level of radical scavenging activity (Nanjo *et al.*, 1996). Acetylation of the hydroxyl groups in the B ring (3', 4'-OH) drastically reduces the scavenging effects of catechin, but modification of the hydroxyl groups in the A ring (5,7- or 3,5,7-OH) hardly showed any decreasing effect. Glucosylation of either the 3' or 4' position in the B ring of epicatechin (EC) markedly decreased its scavenging ability.

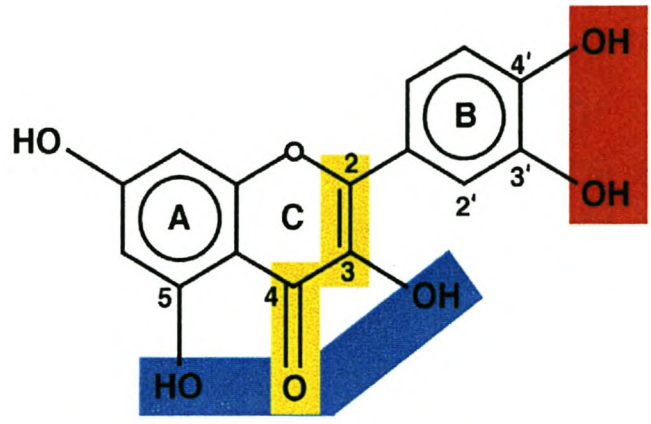


Figure 7 Important criteria for effective radical scavenging by polyphenols include:

the 3',4'-dihydroxy (catechol) configuration in the B-ring which confers high stability to the radical form and participates in electron delocalization;

the 2,3-double bond in conjugation with 4-oxo function in the C-ring which is responsible for electron delocalization from the B-ring; and

the 3- and 5-OH groups which is responsible for maximum radical scavenging potential (Bors *et al.*, 1990).

According to Nanjo *et al.* (1996), studies of catechin metabolism in animals and humans has revealed that more than 80% of the catechins which are absorbed in the body, are in conjugated forms. Therefore, it is most likely that the *in vivo* antioxidant activity of these catechins are less effective than the *in vitro* activity due to the modifications, *i.e.* conjugation of their hydroxyl groups during metabolism. The decrease in the scavenging ability of catechins with decreasing pH-values may be explained in terms of their redox potentials (Nanjo *et al.*, 1996).

Structural features are not the only factor which contribute to the antioxidative and radical scavenging effects of a specific compound; their polarity (accessibility to substrates) (Salah *et al.*, 1995), ionization state (Jovanovic *et al.*, 1994), steric hindrance (Jovanovic *et al.*, 1994) and enzyme inhibition (Robak & Gryglewski, 1988) are also important.

Estimation of antioxidant activity

The protective effect of an antioxidant may be estimated by quantitative determination of the primary and secondary oxidation products of lipid peroxidation (Shahidi & Naczk, 1995). It should be stressed, however, that *in vitro* model systems are only preliminary tests to determine whether an extract or compound possess antiradical or antioxidant potential. Further testing in *in vivo* biological systems is necessary to determine the actual effect of these test compounds on the human body. This is ascribed to the fact that several modifications may occur within the digestive tract before the compound is absorbed or excreted by the body; bioavailability of the compound is therefore crucial for it to exert its positive effect in the human body (Hollman *et al.*, 1997).

The methods that were used in this study to assess the antioxidant and antiradical activities of honeybush tea aqueous extracts, crude phenolic fractions and isolated phenolic compounds, are discussed in this section. No literature is available on the antioxidant and antiradical properties of honeybush tea. However, extensive antioxidant and antiradical studies have been performed in various laboratories worldwide using different model systems (Table 1) on phenolic compounds similar to that isolated from fermented *C. intermedia* by Ferreira *et al.* (1998).

Table 1 Testing of the antioxidant and antiradical efficacies of phenolic compounds similar to the compounds isolated from fermented *C. intermedia* as performed using different model systems in various laboratories worldwide

Compound	Effective concentration and solvent used	Activity	System	Reference
Flavone				
Luteolin	50-100 µM	scavenged 10^{-4} M DPPH$^{\cdot}$ (59%) by H-donation	DPPH$^{\cdot}$ scavenging method	Joyeux *et al.*, 1995
	mol AH:mol DPPH$^{\cdot}$ (1:4); MeOH	potent scavenging of DPPH$^{\cdot}$ (91%)	DPPH$^{\cdot}$ scavenging method	Von Gadow *et al.*, 1997
	50-100 µM; DMSO	significantly decreased MAD production	lipid peroxidation	Joyeux *et al.*, 1995
	50-100 µM; DMSO	significantly decreased LDH leakage	cell membrane disturbance	Joyeux *et al.*, 1995
	10 µg mL^{-1}	effectively decreased LA hydroperoxide formation by H-donation to ROO$^{\cdot}$	LA peroxidation	Torel *et al.*, 1986
	5 x 10^{-5} M; MeOH	potent inhibition of LA peroxidation	β-carotene bleaching method	Von Gadow *et al.*, 1997
	IC_{50} = 5.9 x 10^{-7} M EtOH/Tween 80	strongly inhibited XOD	XOD inhibitory assay	Noro *et al.*, 1983
	–	effectively decreased LDL hydroperoxide formation by H-donation to ROO$^{\cdot}$	LDL oxidation (implicated in pathogenesis of CHD)	Rice-Evans & Miller, 1996
	TEAC = 2.1 ± 0.05 mM	effectively scavenged ABTS$^{\cdot+}$ by H-donation	TAA	Rice-Evans *et al.*, 1996

Table 1 continued

Compound	Effective concentration and solvent used	Activity	System	Reference
Flavanone				
Eriodictyol	10 - 35 µM	potent scavenging of $O_2^{\cdot-}$ (60 - 80%)	XA/XOD	Haraguchi *et al.*, 1996
	10 µM	effective inhibition (84%)	rat liver microsomal lipid peroxidation	Haraguchi *et al.*, 1996
	10 µM	potent inhibition (90%)	rat liver mitochondrial lipid peroxidation	Haraguchi *et al.*, 1996
	100 µM	potent inhibition of CD hydroperoxides (90%) - stronger than TOC	LA peroxidation (thiocyanate method)	Miyake *et al.*, 1997a
	100 µM; DMSO	potent inhibition (86%) of MAD formation - stronger than TOC	rabbit erythrocyte membrane ghost system	Miyake *et al.*, 1997a
	100 µM; DMSO	potent inhibition (> 90%) of lipid peroxidation - stronger than TOC	AAPH-induced liposome peroxidation (TBARS)	Miyake *et al.*, 1997b
Flavanone				
Naringenin	1 mM	scavenges 36% $OH^{\cdot}$ relative to the control	UV photolysis of H_2O_2	Husain *et al.*, 1987
	100 µM	poor inhibition (27%) of MAD formation	rabbit erythrocyte membrane ghost system	Miyake *et al.*, 1997a
	TEAC = 1.53 ± 0.05 mM	scavenged $ABTS^{\cdot+}$ by H-donation	TAA	Rice-Evans *et al.*, 1996

Table 1 continued

Compound	Effective concentration and solvent used	Activity	System	Reference
Flavanone				
Hesperetin	100 µM	moderate inhibition (50%) of MAD formation	rabbit erythrocyte membrane ghost system	Miyake *et al.*, 1997a
	100 µM; DMSO	effective inhibition (60%) of lipid peroxidation	AAPH-induced liposome peroxidation (TBARS)	Miyake *et al.*, 1997b
	TEAC = 1.37 ± 0.08 mM	scavenged $ABTS^{\cdot+}$ by H-donation	TAA	Rice-Evans *et al.*, 1996
	–	poor inhibition of CD formation	lipoprotein oxidation model	Vinson *et al.*, 1995
Flavanone				
Hesperidin	100 µM	moderate inhibition (50%) of lipid peroxidation	LA peroxidation (thiocyanate method)	Miyake *et al.*, 1997b
	100 µM; DMSO	moderate inhibition (50%) of lipid peroxidation	AAPH-induced liposome peroxidation (TBARS)	Miyake *et al.*, 1997b
	100 µM	poor inhibition (35%) of MAD formation	rabbit erythrocyte membrane ghost system	Miyake *et al.*, 1997a
	TEAC = 1.08 ± 0.04 mM	scavenged $ABTS^{\cdot+}$ by H-donation	TAA	Rice-Evans *et al.*, 1996
	1000 µM; DMSO	no inhibition of lipid peroxidation	mouse liver homogenate	Yuting *et al.*, 1990
Xanthones				
mangiferin	50 µM; DMSO	rapidly scavenged $DPPH^{\cdot}$ by H-donation	$DPPH^{\cdot}$ scavenging method	Sato *et al.*, 1992
	50 µM; DMSO	microsomes pretreated with mangiferin: strong inhibition of MAD formation; no pretreatment: no inhibition of MAD	enzymatic microsomal lipid peroxidation (NADPH- or $Fe3^{+}$-induced)	Sato *et al.*, 1992
	200 µM; DMSO	no scavenging of $ROO^{\cdot}$	LA peroxidation	Sato *et al.*, 1992

Table 1 continued

Compound	Effective concentration and solvent used	Activity	System	Reference
Isoflavones				
formononetin	$IC_{50} > 10^{-3}$ mol L^{-1}; EtOH or DMSO in H_2O	poor inhibition of MAD formation	microsomal lipid peroxidation	Jha *et al.*, 1985
Phenolic acid				
4-coumaric acid	TEAC = 2.22 ± 0.06 mM	effectively scavenged $ABTS^{\bullet+}$ by H-donation	TAA	Rice-Evans *et al.*, 1996
	MeOH	very poor $DPPH^{\bullet}$ scavenging	$DPPH^{\bullet}$ scavenging method	Brand-Williams *et al.*, 1995
	mol AH:mol $DPPH^{\bullet}$ (1:2); MeOH	very poor $DPPH^{\bullet}$ scavenging (8%)	$DPPH^{\bullet}$ scavenging method	Von Gadow *et al.*, 1997
	< 1 mg mL^{-1}; MeOH	no activity	$DPPH^{\bullet}$ scavenging method	Goupy *et al.*, 1999
	5 x 10^{-5} M; MeOH	moderate inhibition of LA peroxidation	β-carotene bleaching method	Von Gadow *et al.*, 1997
	< 1 mg mL^{-1}; MeOH	moderate inhibition of LA peroxidation	β-carotene bleaching method	Goupy *et al.*, 1999
	< 1 mg mL^{-1}; MeOH	weak inhibition	Lipoxygenase activity	Goupy *et al.*, 1999
	IC_{50} = 4 μM; gentle sonication in heated H_2O	relatively poor scavenging of $ROO^{\bullet}$	LDL lipid peroxidation	Castelluccio *et al.*, 1995
	0.02% (m/m) AH in lard	very poor antioxidant activity	Rancimat method	Von Gadow *et al.*, 1997

Alphabetical list of abbreviations:

AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride
$ABTS^{\bullet+}$ = 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation
AH = antioxidant
CD = conjugated diene
CHD = coronary heart disease
DMSO = dimethyl sulphoxide
$DPPH^{\bullet}$ = 1,1-diphenyl-2-picrylhydrazyl radical
EtOH = ethanol
H = hydrogen
H_2O_2 = hydrogen peroxide
IC_{50} = [AH] giving 50% inhibition
LA = linoleic acid
LDH = lactate dehydrogenase
LDL = low-density lipoprotein
MAD = malonaldehyde
MeOH = methanol
$O_2^{\bullet-}$ = superoxide anion radical
$OH^{\bullet}$ = hydroxyl radical
$ROO^{\bullet}$ = peroxyl radical
TAA = total antioxidant activity
TBARS = thiobarbituric acid reactive substances
TEAC = Trolox equivalent antioxidant concentration
TOC = α-tocopherol
UV = ultraviolet
XA = xanthine
XOD = xanthine oxidase

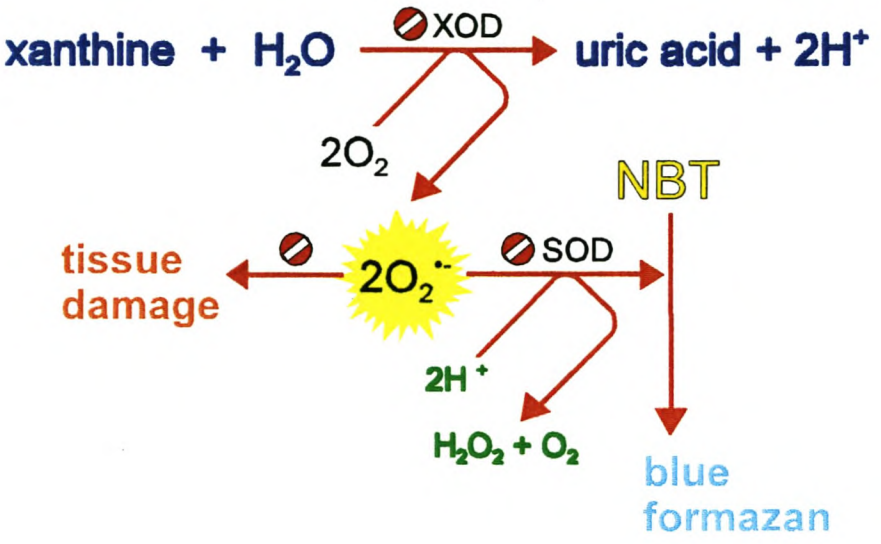
None of the honeybush tea polyphenols have previously been tested by the methods described in this review, except for mangiferin (Sato *et al.*, 1992), luteolin (Joyeux *et al.*, 1995; Von Gadow *et al.*, 1997) and 4-coumaric acid (Brand-Williams *et al.*, 1995; Von Gadow *et al.*, 1997 and Goupy *et al.*, 1999) that were tested for their H-donating ability towards DPPH$^{\bullet}$ (Table 1). Eriodictyol was tested for its $O_2^{\bullet-}$ scavenging ability, but $O_2^{\bullet-}$ was generated enzymatically in a xanthine/xanthine oxidase (XOD) system (Haraguchi *et al.*, 1996).

Dimethyl sulphoxide (DMSO) as a solvent

Dimethyl sulphoxide (DMSO) is widely used as a solvent in order to dissolve compounds and fractions that otherwise poses a problem. Extracts and isolated compounds from *Eucalyptus rostrata* were dissolved in DMSO for testing in the SOD mimic, XOD inhibitory and rabbit erythrocyte ghost membrane assays (Okamura *et al.*, 1993). Sato *et al.* (1992) used DMSO to dissolve the xanthone mangiferin for testing in the DPPH$^{\bullet}$ scavenging method. According to Basaga *et al.* (1997), rosemary extract solubilized in DMSO did not interfere with the experimental system for testing its scavenger activity towards enzymatically generated $O_2^{\bullet-}$. Yuting *et al.* (1990) dissolved quercetin, hesperidin and other flavonoids in DMSO for testing their inhibitory action towards lipid peroxidation in mouse liver homogenate. Quercetin, naringenin, hesperidin and other flavonoids were dissolved in DMSO (Iio *et al.*, 1985), as were tannins and related polyphenols (Hatano *et al.*, 1990), for testing in a XOD inhibitory assay. Saleh *et al.* (1998) dissolved flavonoids (eg. genistein) in DMSO followed by dilution in a salt solution, prior to a chemiluminescence assay.

Xanthine oxidase (XOD) inhibitory assay

Xanthine oxidase (XOD, EC 1.1.3.22), an oxidative enzyme, catalyses uric acid formation from xanthine. A consequence of this process is the generation of $O_2^{\bullet-}$ which is an inducer of oxidative damage, such as lipid peroxidation in living tissues (Gutteridge & Halliwell, 1994; Hatano *et al.*, 1990; Tramper, 1987) (Fig. 8).



Potential sites of inhibition by honeybush polyphenols

Figure 8 The formation of uric acid from xanthine in the presence of xanthine oxidase (XOD) with the consequent generation of superoxide anion radicals ($O_2^{\bullet-}$) (modified from Ricardo da Silva *et al.*, 1991). Potential sites of inhibition by antioxidant substances (eg. polyphenolic compounds from honeybush tea) include scavenging of $O_2^{\bullet-}$ and inhibition of the antioxidant enzymes, XOD and superoxide dismutase (SOD).

Superoxide anion radicals ($O_2^{\bullet-}$) are normally assayed by *in vitro* generation in a xanthine-XOD system in the presence of nitroblue tetrazolium (NBT), which is then reduced by $O_2^{\bullet-}$ with the subsequent formation of blue formazan (Robak & Gryglewski, 1988) (Fig. 8). It became evident that the inhibitory effect of various types of tannins and related polyphenols on lipid peroxidation is not exclusively due to their radical scavenging action towards $O_2^{\bullet-}$, but could also be as a result of their inhibitory effect towards the XOD enzyme (Hatano *et al.*, 1990; Okamura *et al.*, 1993). Test compounds that inhibit XOD result in the formation of less blue formazan as less $O_2^{\bullet-}$ is available to reduce NBT (Hatano *et al.*, 1990; Okamura *et al.*, 1993). In such cases it is advisable to rather use a non-enzymatic method for the generation of $O_2^{\bullet-}$ (Robak & Gryglewski, 1988).

Superoxide anion radical ($O_2^{\bullet-}$) scavenging method

During O_2 metabolism, the reduction of O_2 to H_2O results in the formation of $O_2^{\bullet-}$. These reactive intermediates are involved in oxidative damage to important biological molecules, i.e. lipid peroxidation of membrane lipids (Gutteridge & Halliwell, 1994; Hatano *et al.*, 1990). The generation of $O_2^{\bullet-}$ is either achieved in an enzymatic (xanthine-XOD) (Fig. 8) or a non-enzymatic (β-NADH/PMS) system (Robak & Gryglewski, 1988; Paoletti & Mocali, 1990). For the purpose of the present study, non-enzymatic generation of $O_2^{\bullet-}$ will be discussed. Phenazine methosulphate (PMS) has the ability to couple β-NADH with tetrazolium salts, thereby reducing NBT. The reduction of NBT, with the subsequent formation of blue formazan is measured spectrophotometrically at 560 nm (Robak & Gryglewski, 1988; Ponti *et al.*, 1978). The reactions are as follows (Ponti *et al.*, 1978):

$$\mathbf{NADH + H^+ + PMS \rightarrow NAD^+ + PMSH_2}$$

$$\mathbf{PMSH_2 + 2O_2 \rightarrow 2O_2^{\bullet-} + 2H^+ + PMS}$$

$$\mathbf{NBT^{2+} + 2Cl^- + 4O_2^{\bullet-} + 4H^+ \rightarrow diformazan + 4O_2 + 2HCl}$$

Superoxide dismutase (SOD) catalyzes the following dismutation reaction to neutralize the harmfull $O_2^{\bullet-}$ (McCord & Fridovich, 1969; Paoletti & Mocali, 1990):

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$$

One unit of SOD activity is the quantity of SOD required to inhibit the rate of reduction of NBT by 50% (McCord & Fridovich, 1969). Superoxide dismutase (SOD) prepared from bovine erythrocytes consists of specific activity of *ca*. 5000 units/mg protein (Boehringer Mannheim, Germany), whereas McCord & Fridovich (1969) estimated the activity at *ca*. 3000 units/mg protein.

Evaluation of H-donating ability through DPPH$^\bullet$ radical scavenging

One of the most important inhibitory reactions of peroxidation of fats and oils is the donation of H^+ or electrons by an antioxidant towards free radicals generated during the propagation phase of lipid peroxidation (Kurechi & Kato, 1982), thereby converting these radicals into more stable species, and thus breaking the radical chain reaction (Eriksson & Na, 1995).

The chain-breaking action of an antioxidant (AH) acting as a H-donor in order to scavenge lipid alkoxyl (RO$^\bullet$) and peroxyl (ROO$^\bullet$) radicals, is schematically represented by the following (Rice-Evans, 1995):

$$ROO^\bullet + AH \rightarrow ROOH + A^\bullet$$
$$RO^\bullet + AH \rightarrow ROH + A^\bullet$$

The free radical DPPH$^\bullet$, acting as a H acceptor for antioxidants, simulates the chain-breaking reaction between antioxidants and ROO$^\bullet$ (Kurechi *et al.*, 1980). Antioxidant compounds are allowed to react with this stable radical in a methanol solution. In its radical form, DPPH$^\bullet$ absorbs maximally at 515 nm, but upon reduction by an antioxidant or radical species (R$^\bullet$) the absorption disappears.

The reduction of DPPH$^{\bullet}$ by an antioxidant is followed by monitoring the decrease in its absorbance at this characteristic wavelength during the reaction (Brand-Williams *et al.*, 1995):

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$
$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$$

Inhibition of linoleic acid peroxidation

Lipid oxidation is not only one of the major causes influencing food quality, thereby making it unacceptable for human consumption (Labuza, 1971), but it is also related to tissue damage associated with various human diseases (Davies, 1995). The abstraction of H from PUFAs results in the formation of free-radical intermediates and rearrangement of double bonds to form conjugated diene fatty acid hydroperoxides (CDOOH) (Porter *et al.*, 1984). The presence of conjugated dienes (CD) in a biological system is evident of an ongoing lipoperoxidative process (Corongiu & Banni, 1994). Similarly, a well-defined model system using linoleic acid as a substrate, is used to measure the formation of CD compounds (linoleic acid hydroperoxides) as indicators of lipid peroxidation (Lingnert *et al.*, 1979). Conjugated dienes (CD) are used as a marker of lipid peroxidation due to the characteristic absorption of dienes at 234 nm that is detected by ultraviolet (UV) spectrophotometry (Lingnert *et al.*, 1979). Parameters, such as temperature, incubation time and concentration of linoleic acid in the emulsion, are optimized (Lingnert *et al.*, 1979).

Although linoleic acid is a relatively minor component in food systems, this simple model system will contribute to understand the fundamentals underlying lipid oxidation (Ponginebbi *et al.*, 1999). However, results obtained from the linoleic acid model system may not be extrapolated directly into a food system or to the human body, as the mechanism of antioxidant activity in the one system does not necessarily represent the activity in the other system (Warner, 1997). The addition of Tween 20, a nonionic surfactant, improves the dispersion of linoleic acid in the reaction mixture, thereby improving the spectrophotometric recording of linoleic acid peroxidation (Surrey, 1964). An oil-in-water emulsion consisting of three components, i.e. the dispersing phase

(H_2O), the dispersed phase (linoleic acid) and the interface (Tween 20), was thus prepared. The effectiveness of an antioxidant at the interface of oil droplets, a potential starting point for oxidation (Coupland *et al.*, 1996), depends to a great extent on its polarity (Frankel *et al.*, 1994).

Conclusion

Oxidative deterioration in foodstuffs and the human body results from insufficient antioxidant defence. Several studies have hitherto shown the benefits of a plant-based diet to promote health and well-being. The antioxidant activity of naturally occurring plant compounds such as polyphenols has an important implication in scavenging harmful free radicals and in retarding lipid peroxidation in the body. Polyphenols are also considered as possible safer alternatives to synthetic food additives. The antioxidant activity of polyphenols should be evaluated using a combination of model systems to understand their role as free radical scavengers.

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Chapter 3

Antioxidant and antiradical activities of aqueous extracts of different *Cyclopia* species as affected by fermentation

Abstract

The influence of fermentation on the ability of aqueous extracts from *Cyclopia intermedia*, *C. genistoides*, *C. maculata*, *C. sessiliflora* and *C. subternata* to scavenge 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) radicals, was quantified. In addition, their inhibitory effect towards linoleic acid peroxidation was determined. All tests were performed on an equal mass basis. Aqueous extracts of unfermented material of *C. sessiliflora* with the highest total polyphenol and flavonoid contents, showed the most effective hydrogen-donating (H-donating) ability to $DPPH^{\bullet}$ and scavenging of $O_2^{\bullet-}$ ($P \leq 0.05$). Fermented *C. subternata* showed the weakest H-donating ability, followed closely by fermented *C. maculata* and *C. intermedia*. This phenomenon is partially attributed to their soluble solids containing the lowest total polyphenol contents ($P \leq 0.05$). Fermented *C. maculata* and *C. intermedia* also showed the weakest $O_2^{\bullet-}$ scavenging abilities ($P \leq 0.05$). Fermentation decreased H-donating and $O_2^{\bullet-}$ scavenging abilities of aqueous extracts ($P \leq 0.05$), possibly resulting from the oxidation of the phenolic compounds during fermentation. On the contrary, fermentation did not significantly affect inhibition of linoleic acid peroxidation ($P \geq 0.05$), except for unfermented *C. intermedia* being a slightly weaker inhibitor than its fermented equivalent ($P \leq 0.05$). Overall, aqueous extracts from fermented and unfermented *C. genistoides* showed the most effective inhibition of linoleic acid peroxidation, whereas unfermented *C. intermedia* exhibited the least effective inhibition of linoleic acid peroxidation.

Introduction

Honeybush tea is a traditional South African herbal beverage which has been consumed locally for many centuries, not purely for enjoyment, but probably due to the so-called health-giving properties (Watt & Breyer-Brandwijk, 1962). The increasing popularity of honeybush tea, both locally and internationally, is in accordance with the world-wide increased consumption of herbal infusions (Hasler, 1998). Processing (fermentation) of the plant material is essential to develop the desired sensory properties. Of the more than 20 species of honeybush tea belonging to the indigenous legume family Fabaceae (Leguminosae), only *Cyclopia intermedia* and to a lesser extent *C. subternata* are processed for marketing. *Cyclopia genistoides, C. sessiliflora* and *C. maculata* are currently being evaluated for commercial cultivation at the ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa.

The flavanones, flavones, isoflavones, coumestans, xanthones and a phenolic acid present in fermented material of *C. intermedia* are associated with a vast diversity of pharmacological properties (Ferreira *et al.*, 1998). Some of these compounds, such as the flavone luteolin and flavanones eriodictyol, hesperetin and naringenin, have shown antioxidant and antiradical potential in various *in vitro* and *in vivo* model systems (Sato *et al.*, 1992; Joyeux *et al.*, 1995; Haraguchi *et al.*, 1996; Rice-Evans *et al.*, 1996; Miyake *et al.*, 1997). Although it is expected, from previous experience (Wanasundara & Shahidi, 1998), that honeybush tea may display similar properties, none of the *Cyclopia* species have been evaluated for their antioxidant and antiradical properties. It is also possible that the variation in species phenolic profiles (De Nysschen *et al.*, 1996) and the fact that the fermentation process results in a decrease in the total polyphenol content (Du Toit & Joubert, 1998), could have a direct impact on the antioxidant status of honeybush tea.

It must be taken into consideration that the successful marketing of honeybush tea as a health beverage in a competitive herbal tea and antioxidant-rich beverage market will depend to a large extent on the antioxidant properties ascribed to it. The aim of this investigation was therefore to evaluate the *in vitro* antioxidant and free radical scavenging abilities of aqueous extracts of different *Cyclopia* species and the influence of fermentation on these species. The superoxide anion radical ($O_2^{\bullet-}$) scavenging

activity, hydrogen-donating (H-donating) ability to 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radicals, and inhibitory effect towards linoleic acid peroxidation of fermented and unfermented material of *C. intermedia*, *C. subternata*, *C. genistoides*, *C. maculata* and *C. sessiliflora* were evaluated. The xanthine oxidase (XOD) inhibitory assay was used to evaluate the inhibitory effect of aqueous extracts from fermented and unfermented material of *C. intermedia* on the activity of XOD.

Materials and methods

Chemicals

Superoxide dismutase (SOD, EC 1.15.1.1) (from bovine erythrocytes, 5000 units mg^{-1}), 4-nitroblue tetrazolium chloride (NBT) and XOD (xanthine:oxygen oxireductase; EC 1.1.3.22) (from cow's milk, 20 units mL^{-1}) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Phenazine methosulphate (PMS) (N-methyl-dibenzo-pyrazine methyl sulphate salt), β-nicotinamide adenine dinucleotide (β-NADH), reduced form disodium salt (98%), xanthine (2,6-dihydroxypurine) (99 - 100%), 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) (*ca.* 90%), linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid) (≥99%), polyoxyethylene-sorbitan monolaurate (Tween 20), butylated hydroxytoluene (BHT) (2,[6]-di-tert-butyl-*p*-cresol) and butylated hydroxy-anisole (BHA) (2[3]-*t*-butyl-4-hydroxyanisole) were purchased from Sigma Chemical Co. (St Louis, USA). Sodium dihydrogen phosphate 2-hydrate ($NaH_2PO_4.2H_2O$; purified), *di*-sodium hydrogen phosphate 12-hydrate crystalline ($Na_2HPO_4.12H_2O$; extra pure), *di*-potassium hydrogen phosphate (anhydrous K_2HPO_4; extra pure), potassium dihydrogen orthophosphate (anhydrous KH_2PO_4), sodium carbonate (anhydrous Na_2CO_3; analytical reagent (AR) grade), ethylene-diamine-tetraacetic acid disodium salt (EDTA) (AR) and Folin Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, Germany). Gallic acid (chemically pure crystalline) was purchased from Riedel-de Haën (AG Seelze-Hannover). Methanol (AR; 99.8%), formaldehyde (37 - 40%, m/v) and dimethyl sulphoxide (DMSO) were supplied by BDH Chemicals Ltd (Poole, England). Hydrochloric acid (HCl), potassium hydroxide (KOH; AR) and sodium hydroxide (NaOH) pellets were supplied by Saarchem (Johannesburg, South Africa).

The deionised water used in this study refers to water treated sequentially with carbon, RO (reverse osmosis) and with a mixed-bed deioniser to obtain a conductivity of 0.1 μs cm^{-1} using a Modulab Water Purification System (Continental Water Systems Corporation, Texas, USA).

Apparatus

All spectrophotometric measurements were carried out in a 1 cm path length quartz cell, using a Beckman DU-65 (UV/VIS) spectrophotometer, except when noted otherwise. Data from time assays were recorded with Beckman Data Capture Software.

Harvesting and processing of plant material

Cyclopia subternata (*ex* Langkloof), *C. genistoides* (*ex* Rondeberg, West Coast), *C. sessiliflora* (*ex* Riversdal) and *C. maculata* (*ex* Genadendal) were harvested during May 1997 at the experimental plantation at Genadendal, Western Cape Province, while *C. intermedia* (*ex* Langkloof) was harvested in June 1997 from natural plant populations in the mountainous area of Vallei Vrugteplase, Louterwater in the Langkloof, Eastern Cape Province. The experimental plantings of Genadendal were established in 1995 for all species, except that of *C. maculata* which was established in 1996. Of each species, four separate bundles (*ca.* 5 - 8 kg each) were harvested representing four replicates. The shoots of 22 ± 12 plants were pooled to form a bundle. The variation in the amount of plant material is due to the sizes of plants varying between species. The tea material was processed according to the standardised processing procedure of Du Toit & Joubert (1998). The shoots of each species were cut into small pieces (≤ 4 mm) by a modified fodder cutter. The cut plant material of each bundle (*ca.* 2 kg) was moistened with deionised water (4:1), followed by fermentation (70°C for 60 h) in a laboratory incubator. The fermented material was dried (40°C for 12 h) in a drying tunnel (Decon Humidifier, Continental Fan Works CC., Cape Town, South Africa). The unfermented material was prepared by drying the remaining material of each bundle directly after cutting in the drying tunnel (40°C for 12 h). The dried tea (< 10% moisture content; wet basis) was sieved with an Endecotts test sieve (2 mm) (London,

England). A fraction containing leaves and small pieces of stems (≤ 2 mm), and a coarser fraction containing woody material and uncut leaves (> 2 mm), were obtained. The following abbreviated notations will be used to describe the species and their fermentation status: Ses(U) (unfermented *C. sessiliflora*); Ses(F) (fermented *C. sessiliflora*); Gen(U) (unfermented *C. genistoides*); Gen(F) (fermented *C. genistoides*); Mac(U) (unfermented *C. maculata*); Mac(F) (fermented *C. maculata*); Sub(U) (unfermented *C. subternata*); Sub(F) (fermented *C. subternata*); Int(U) (unfermented *C. intermedia*) and Int(F) (fermented *C. intermedia*).

Preparation of aqueous extracts

Aqueous extracts were prepared from fermented and unfermented material of each of the four bundles of the different species. Freshly boiled deionised water (500 mL) was poured onto tea leaves (*ca.* 20 g) (≤ 2 mm) followed by steeping for exactly 5 min in a sealed vacuum flask. The extract was decanted through a strainer directly into a volumetric flask (500 mL), followed by rapid cooling in an ice bath to room temperature. The extract was filtered through Whatman No. 54 filter paper, after adjusting the volume of the cooled extract to 500 mL with deionised water. Aliquots of the filtrates (*ca.* 25 mL) were stored in specimen containers at -18°C until analysed.

Soluble solid, total polyphenol and flavonoid contents of aqueous extracts

The soluble solid content of the filtrates was determined gravimetrically. Duplicate aliquots of each filtrate (20 mL) were evaporated to dryness in nickel moisture dishes on a steam bath (Baird & Tatlock Ltd, Chadwell Heath, Essex, London), followed by drying under vacuum (70°C for 16 h). The concentration of soluble solids in the aqueous extracts was expressed as g soluble solids 100 mL^{-1} extract. The total polyphenol and flavonoid contents of aqueous extracts were determined according to the Folin Ciocalteu method of Singleton & Rossi (1965) and Kramling & Singleton (1969), respectively. Gallic acid was used as standard and the concentrations of total polyphenols and flavonoids were accordingly expressed as g gallic acid equivalents (GAE) 100 mL^{-1} extract or 100 g^{-1} soluble solids.

Hydrogen-donating ability (DPPH$^{\bullet}$ scavenging)

The H-donating ability of aqueous extracts prepared from fermented and unfermented *Cyclopia* species was evaluated using the stable free radical, DPPH$^{\bullet}$ in a methanol solution, according to a slightly modified version of the method of Brand-Williams *et al.* (1995). Reduction of DPPH$^{\bullet}$ to DPPH-H was measured as a decrease in absorbance at 515 nm. Although DPPH$^{\bullet}$ is considered as a relatively stable free radical, its solution slowly deteriorates. It was therefore shielded from light during the assay by covering with aluminium foil (Blois, 1958) and storing at 0°C for not more than two days. A dilution series of each tea extract was prepared to give *ca.* 10 - 90% inhibition of DPPH$^{\bullet}$.

Controls consisted of deionised water (50 µL) and DPPH$^{\bullet}$ solution (2 mL) (C_{DPPH} = 3 x 10^{-5} mol L^{-1}), whereas samples contained diluted tea extracts (50 µL) as substitute for the deionised water. Blank samples consisted of the diluted tea extracts (50 µL) and pure methanol (2 mL) instead of the DPPH$^{\bullet}$ solution. Controls, samples and blanks were prepared in screw-capped reaction vials and the absorbances were measured at 515 nm after a 2 h-incubation period in the dark at room temperature. The absorbance values of the blanks were subtracted from the absorbance values obtained for the samples.

Absorbance values at 515 nm are proportional to the amount of residual DPPH$^{\bullet}$ at steady state (Saint-Cricq de Gaulejac, 1999). For the purpose of this study it was assumed that a steady state was reached after the 2 h-incubation period. The scavenging (%) of the DPPH$^{\bullet}$ initially present in the reaction mixture, which is inversely proportional to the absorbance values at 515 nm, was calculated at t = 2 h for each test sample using the following formula:

$$\textit{Scavenging (\%) of DPPH}^{\bullet} = \frac{A_c - A_s}{A_c} \times 100$$

where: A_c = Absorbance (λ = 515 nm) of control after 2 h

A_s = Absorbance (λ = 515 nm) of sample after 2 h

The scavenging (%) of the initial DPPH$^{\bullet}$ was plotted as a function of the concentration of soluble solids in 50 µL of the test sample in the reaction mixture. The H-donating ability of the tea extracts was evaluated in terms of their respective EC_{50} values (i.e. Efficient Concentration of antioxidant (µg) necessary to neutralize the initial DPPH$^{\bullet}$ by 50%). The EC_{50} values were calculated following linear regression of the direct proportional part of each separate curve. EC_{50} values were normalized to compensate for the small variation which may occur in the concentration of DPPH$^{\bullet}$ in subsequent assays. The exact amount of DPPH$^{\bullet}$ in the reaction mixture was calculated using a calibration curve giving the direct proportional correlation between absorbance (λ = 515 nm) and different concentrations of DPPH$^{\bullet}$ in methanol (mol DPPH$^{\bullet}$ L^{-1}) prepared from a stock solution (3.043368×10^{-5} mol DPPH L^{-1}).

XOD inhibitory assay

The inhibitory effect of aqueous extracts from fermented and unfermented *C. intermedia* on the activity of XOD was measured. A combination of the methods by Hatano *et al.* (1990) and Noro *et al.* (1983), modified from the original method reported by Kalckar (1947), was used. The concentration of tea soluble solids in the final reaction mixture (0.20 g L^{-1}) was similar to that tested in the $O_2^{\bullet-}$ scavenging method. Controls were prepared by pre-incubating XOD solution (0.04 unit mL^{-1}; 100 µL) in 0.1 M phosphate buffer (pH 7.5; 1.5 mL) for 1 min in a water bath at 25°C. For testing the inhibitory effect of tea extracts towards XOD, 500 µL of tea extract was pre-incubated with XOD (0.04 unit mL^{-1}; 100 µL) in 0.1 M phosphate buffer (pH 7.5; 1 mL) for 1 min at 25°C. An aqueous solution of xanthine (0.8 mM; 500 µL) was added to the reaction mixture followed by stirring with a plastic stirring rod. Xanthine, which is insoluble in small volumes of water, was prepared fresh daily by dissolving the required amount in a few drops of sodium hydroxide (1 M) followed by diluting with 0.1 M phosphate buffer (pH 7.5). The XOD solution was prepared fresh daily and kept on ice during the assay as recommended by L'Abbé & Fischer (1990). Spectrophotometric readings, initiated exactly 15 s after addition of the xanthine solution, was taken every second for 5 min in a thermostated cell holder (25°C) at 295 nm. The increase in absorbance is indicative of uric acid formation (Robak & Gryglewski, 1988).

For each tea extract tested, the rate of inhibition (%) of XOD was calculated as follows (Costantino *et al.*, 1992):

$$\textit{Inhibition (\%) of XOD} = \left(1 - \frac{\Delta A_{test}}{\Delta A_{control}}\right) \times 100$$

where: $\Delta A_{control}$ = the change in absorbance over 5 min due to the activity of the XOD enzyme without test material

ΔA_{test} = the change in activity of the XOD enzyme as affected by test material.

Superoxide anion radical ($O_2^{\bullet-}$) scavenging ability

Aqueous extracts prepared from fermented and unfermented *Cyclopia* species were studied as potential scavengers of $O_2^{\bullet-}$ using the non-enzymatic (β-NADH/PMS) method of Robak & Gryglewski (1988). Superoxide anion radicals ($O_2^{\bullet-}$) were generated in an incubation mixture (2 mL) with final concentrations of 156 µM β-NADH, 30 µM PMS and 630 µM NBT in 0.1 M sodium phosphate buffer at physiological pH (pH 7.4). The formation of blue formazan, as the reduction product of NBT by $O_2^{\bullet-}$, was measured spectrophotometrically (λ = 560 nm) at room temperature every 2 s over a 2 min period.

The ability of the test material to scavenge $O_2^{\bullet-}$ was evaluated in comparison to a control (without inhibitor). Superoxide dismutase (SOD), an antioxidant enzyme, was used as reference compound. All reagents were prepared freshly with chilled sodium phosphate buffer (0.1 M; pH 7.4), except NBT for which buffer at room temperature was used. During the assay, reagents were kept on ice in vessels shielded from light by covering with aluminium foil (Ponti *et al.*, 1978). Plastic disposable cuvettes were used as recommended by Green & Hill (1984), as formazan, which is insoluble in water, tends to adhere to glass.

Stock solutions of 2.0 g L^{-1} and 0.40 g L^{-1} were prepared of the tea soluble solids and SOD (in buffer), respectively. The stock solutions were further diluted to obtain five different concentrations, giving within 20 to 80% inhibition of NBT reduction. Controls were prepared by pipetting 500 µL each of 0.1 M phosphate buffer (pH 7.4) and the

NBT (2,52 x 10^3 µM) and β-NADH (624 µM) solutions into a disposable cuvette, after which the reaction was initiated by the addition of 500 µL PMS (120 µM).

For preparation of samples, the 500 µL of phosphate buffer in the reaction mixture was replaced with an equivalent volume of test sample. Blank samples were prepared for each test sample, containing 500 µL of phosphate buffer instead of PMS, of which the absorbance value at 560 nm was subtracted from the absorbance values of test samples over time. A separate control was prepared for each series of dilutions of a test sample. The inhibition (%) of NBT reduction by each test sample was calculated at t = 2 min using the following formula:

$$\textit{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where: A_c = Absorbance of control (λ = 560 nm) after 2 min

A_s = Absorbance of sample (λ = 560 nm) after 2 min

A log dose-inhibition curve was plotted of inhibition (%) of NBT reduction as a function of the log amount of test sample mL^{-1} reaction mixture (Costantino *et al.*, 1992). From each separate log dose-inhibition curve an IC_{50} value (i.e. the concentration of soluble solids or SOD required to inhibit NBT reduction by 50%) was obtained (Costantino *et al.*, 1992; Robak & Gryglewski, 1988). IC_{50} values were calculated by solving the antilog of x following linear regression (y = a + bx), with y substituted as 50. For each species tested an average IC_{50} value, which is the mean of four replicates (n = 4), was calculated. For SOD, the average IC_{50} value is the mean of three replicates (n = 3).

Inhibition of linoleic acid peroxidation

The effects of species and fermentation on the antioxidant activity of aqueous extracts of honeybush tea were evaluated according to Lingnert *et al.* (1979). This well-defined model system uses linoleic acid as substrate and measures the formation of conjugated diene (CD) or linoleic acid hydroperoxides (ROOH) (Lingnert *et al.*, 1979), the major initial reaction products of fatty acids with oxygen (Gray, 1978), as an indication of the degree of oxidation of the sample.

The substrate (10 mM linoleic acid emulsion) consisted of linoleic acid (*ca.* 0.2804 g) and Tween 20 (0.5%, v/v), as surfactant, in chilled potassium phosphate buffer (0.1 M; pH 6.5) (100 mL). The emulsion was prepared using a Vibra-cell high intensity ultrasonic processor (Sonics & Materials Inc., Newtown, USA) equipped with a titanium alloy standard probe (⌀ 13 mm) for 1 min at 100% frequency. Since linoleic acid is very heat sensitive, emulsification was performed in an ice bath. The linoleic acid concentration differed to a certain extent between subsequent assays as a result of difficulty when weighing this viscous substance. The degree of oxidation of the linoleic acid emulsion was determined spectrophotometrically prior to use according to Lingnert *et al.* (1979). All glass vials and test tubes were soaked in an EDTA (0.5%, m/v) solution (Farag *et al.*, 1989) for *ca.* 21 h to chelate any metal ions, followed by thoroughly rinsing of glassware with deionised water and drying at 80°C prior to use.

Controls and samples were prepared by respectively vortexing (Vortex Genie-2, Scientific Industries, Inc., New York, USA) deionised water or aqueous honeybush tea extract (200 µL) with 10 mM linoleic acid emulsion (4 mL) in screw-capped glass vials (*ca.* 40 mL capacity). The inhibition (%) of linoleic acid peroxidation by tea extracts was compared to that of BHA and BHT dissolved in DMSO, for which a separate set of controls consisting of DMSO (200 µL) and 10 mM linoleic acid emulsion (4 mL), were prepared. All tea extracts, as well as BHA and BHT, were evaluated on an equal mass (m/v) basis. Stock solutions with final concentrations of 0.20 g L^{-1} were prepared. The concentration of tea soluble solids, BHA and BHT in the final reaction mixture was 9.50 mg L^{-1}. Blanks consisted of test sample (200 µL) and 0.1 M potassium phosphate buffer (4 mL) (pH 6.5). The absorbance values of the blanks were subtracted from that of the samples. All controls, samples and blanks were incubated for 21 h in darkness at 37°C. At time zero and 3 h intervals during the 21 h-incubation period, aliquots (200 µL) were withdrawn from controls, samples and blanks and dissolved in absolute methanol (2 mL), followed by vortexing with 60% (v/v) methanol (6 mL). The calibration mixture consisted of 0.1 M potassium phosphate buffer (200 µL) (pH 6.5), absolute methanol (2 mL) and 60% (v/v) methanol (6 mL).

The peroxidation of linoleic acid resulted in an increase in absorbance at 234 nm which was plotted over time (h). The antioxidant effect of the test samples compared to the control, was evaluated in terms of the rate of linoleic acid peroxidation (see

statistical analysis), and by the inhibition (%) of the CD hydroperoxides generated in the reaction mixture after 21 h, as follows:

$$\textit{Inhibition (\%) of CD hydroperoxide} = \frac{A_c - A_s}{A_c} \times 100$$

where: A_c = Absorbance (λ = 234 nm) of control at 21 h

A_s = Absorbance (λ = 234 nm) of sample at 21 h

Statistical analyses

Analysis of variance (ANOVA) (SAS Release version 6.12) was used to determine whether species and fermentation significantly affected the parameters tested. Pairwise Student's t-tests were used to compare means when an effect was significant ($P \leq 0.05$). For evaluating linoleic acid peroxidation over time, separate lines were fitted for each test sample. Regression analysis was performed on the data. Pairwise student's t-LSD (least significant difference) ($P = 0.05$) was used to test whether intercepts and gradients differed significantly. In cases where differences were not significant, data for these test samples were pooled and a single line fitted.

Results and discussion

Soluble solid, total polyphenol and flavonoid contents of aqueous extracts

The soluble solid, total polyphenol and flavonoid contents of the aqueous extracts prepared from the five different *Cyclopia* species, showed significant differences (Table 1). *Cyclopia sessiliflora* and *C. intermedia* were the only species that showed a higher soluble solid content for the aqueous extracts prepared from the unfermented material, in comparison to the fermented material ($P \leq 0.05$). In the case of *C. genistoides*, *C. maculata* and *C. subternata*, fermentation did not affect the soluble solid contents of the aqueous extracts (Table 1). Overall, the aqueous extract from unfermented *C. sessiliflora* had the highest soluble solid content, followed by fermented

C. sessiliflora, while fermented *C. subternata* contained the lowest concentration of soluble solids ($P \leq 0.05$).

For all five *Cyclopia* species tested, aqueous extracts prepared from the unfermented material showed higher total polyphenol and flavonoid contents ($P \leq 0.05$) than their fermented equivalents (Table 1), even when expressed on a soluble solid basis (Table 2). The decrease in polyphenols during fermentation is attributed, amongst others, to oxidation and possible polymerisation of smaller monomeric compounds, thereby resulting in lower solubility. The highest total polyphenol and flavonoid contents ($P \leq 0.05$) were obtained for aqueous extracts from unfermented *C. sessiliflora*. Aqueous extracts from fermented *C. subternata*, which had the lowest ($P \leq 0.05$) total polyphenol contents, did not differ significantly ($P \geq 0.05$) from fermented *C. maculata* and *C. intermedia.* Aqueous extracts from fermented *C. subternata* and *C. maculata* also had the lowest ($P \leq 0.05$) flavonoid contents. The total polyphenol contents of the aqueous extracts from unfermented *Cyclopia* species, decreased in the order: Ses > Gen $\geq$ Int $\geq$ Mac > Sub, however, for the fermented equivalents the order was: Ses > Gen > Int $\approx$ Mac $\geq$ Sub. The flavonoid content of aqueous extracts from unfermented *Cyclopia* species, decreased in a slightly different order: Ses > Int > Gen > Mac $\approx$ Sub. The decreasing order of the flavonoid content of fermented material was the same as for the unfermented equivalents, except that *C. intermedia* and *C. genistoides* did not differ significantly ($P \geq 0.05$).

When expressed on a soluble solid basis (Table 2), the total polyphenol contents for unfermented *Cyclopia* species, decreased in the order: Ses > Gen > Mac $\approx$ Int $\approx$ Sub, while the order for the fermented equivalents, was as follows: Ses > Gen > Int $\approx$ Mac $\approx$ Sub. The overall order for total polyphenols expressed on a soluble solid basis for unfermented and fermented *Cyclopia* species, was as follows: ses(U) > gen(U) > mac(U) $\approx$ int(U) $\approx$ ses(F) $\approx$ sub(U) > gen(F) > int(F) $\approx$ mac(F) $\approx$ sub(F). In the case of flavonoids expressed on a soluble solid basis (Table 2), the decreasing order for unfermented *Cyclopia* species was: Ses > Int > Gen > Sub $\approx$ Mac, however, for fermented *Cyclopia* species the order was as follows: Ses > Int $\geq$ Gen > Mac $\approx$ Sub.

Table 1 Soluble solid, total polyphenol and flavonoid contents of aqueous extracts of unfermented and fermented *Cyclopia* species

Species	Soluble solids [1,2]		Total polyphenols [1,3]		Flavonoids [1,3]	
	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented
C. sessiliflora	0.597 a	0.519 b	0.196 a	0.121 bc	0.105 a	0.065 c
C. genistoides	0.480 cd	0.479 cd	0.129 b	0.086 e	0.056 d	0.034 e
C. maculata	0.462 de	0.436 e	0.114 c	0.057 f	0.042 e	0.019 f
C. subternata	0.431 ef	0.403 f	0.100 d	0.049 fg	0.040 e	0.017 f
C. intermedia	0.508 bc	0.443 e	0.123 bc	0.061 f	0.081 b	0.037 e

[1] Each value represents the means of four replicates (n = 4). For unfermented and fermented tea, the respective means of the soluble solids, total polyphenols and flavonoids, followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[2] Concentration expressed as g soluble solids 100 mL^{-1} aqueous extract.

[3] Concentration expressed in terms of g GAE 100 mL^{-1} aqueous extract.

Table 2 Total polyphenol and flavonoid contents expressed as percentage of the soluble solids of aqueous extracts of unfermented and fermented *Cyclopia* species

Species	Total polyphenols [1,2]		Flavonoids [1,2]	
	Unfermented	Fermented	Unfermented	Fermented
C. sessiliflora	32.8 a	23.3 c	17.6 a	12.5 c
C. genistoides	26.9 b	18.0 d	11.7 c	7.1 e
C. maculata	24.7 c	13.1 e	9.1 d	4.4 f
C. subternata	23.2 c	12.2 e	9.3 d	4.3 f
C. intermedia	24.2 c	13.8 e	15.9 b	8.4 de

[1] Each value represents the means of four replicates (n = 4) with duplicate analysis. Means for total polyphenols and flavonoids respectively, irrespective of fermentation, that are followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[2] Concentration expressed in terms of g GAE 100 g^{-1} soluble solids.

The overall order for flavonoids expressed on a soluble solid basis for unfermented and fermented *Cyclopia* species, was as follows: ses(U) > int(U) > ses(F) ≈ gen(U) > sub(U) ≈ mac(U) ≥ int(F) ≥ gen(F) > mac(F) ≈ sub(F). The variation observed in the soluble solid, total polyphenol and flavonoid contents for different species, may clarify the differences observed for H-donating and $O_2^{\bullet-}$ scavenging abilities, and inhibition of linoleic acid peroxidation.

Hydrogen-donating ability (DPPH$^{\bullet}$ scavenging)

The H-donating ability of aqueous extracts prepared from different *Cyclopia* species to DPPH$^{\bullet}$ was expressed in terms of EC_{50} values, as defined by Brand-Williams *et al.* (1995), with lower EC_{50} values indicating higher H-donating ability, and *vice versa* (Table 3). All *Cyclopia* extracts reduced DPPH$^{\bullet}$; however, the potency differed significantly between species ($P \leq 0.05$). A typical example is the aqueous extract of fermented *C. sessiliflora* (Fig. 1) that shows that the scavenging (%) of the initial DPPH$^{\bullet}$ increased in a concentration-dependant manner.

The H-donating ability of unfermented *Cyclopia* species decreased in the order: Ses > Int ≈ Mac ≥ Gen ≥ Sub, with EC_{50} values ranging between 7.7 and 13.4 µg soluble solids 50 μL^{-1} aqueous tea extract in the reaction mixture. For fermented *Cyclopia* species, however, the order was slightly different: Ses > Gen > Int ≥ Mac ≥ Sub, with significantly higher EC_{50} values ($P \leq 0.05$) ranging between 11.2 and 31.2 µg soluble solids 50 μL^{-1} aqueous tea extract in the reaction mixture. For all species, aqueous extracts prepared from unfermented honeybush tea was thus more effective in scavenging DPPH$^{\bullet}$ than their fermented equivalents.

This is attributed to the soluble solids of the unfermented species having higher total polyphenol and flavonoid contents than the fermented species (Table 2). Du Toit & Joubert (1998) also reported a significant decrease in the total polyphenol content of honeybush tea extracts during fermentation. The decreasing antiradical effect observed with fermentation for different honeybush tea species, is in all probability due to oxidation of the phenolic compounds during fermentation, resulting in a loss of hydroxyl groups which is essential for effective radical scavenging.

Table 3 Hydrogen-donating ability to DPPH$^{\bullet}$ of aqueous extracts from different *Cyclopia* species as affected by fermentation

Species	EC_{50} [1,2]	
	Unfermented	**Fermented**
C. sessiliflora	7.7 a	11.2 b
C. genistoides	11.9 bc	20.6 e
C. maculata	11.2 b	27.1 fg
C. subternata	13.4 cd	31.2 g
C. intermedia	10.5 b	25.3 f

[1] Each value represents the means of four replicates (n = 4) with duplicate analysis. Means within the table followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[2] Expressed as µg soluble solids 50 μL^{-1} aqueous tea extract in the reaction mixture necessary to decrease initial [DPPH$^{\bullet}$] by 50%.

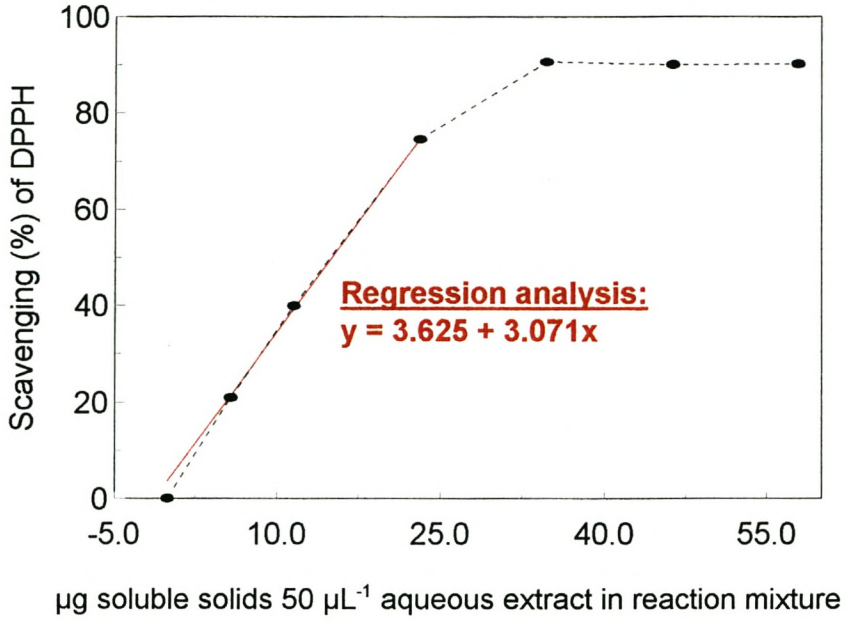

Figure 1 The scavenging (%) of DPPH$^{\cdot}$ plotted as a function of the soluble solid concentration (0 to 57.9 µg soluble solids 50 μL^{-1} aqueous extract) of fermented *C. sessiliflora* in the reaction mixture (EC_{50} = 15.1).

Serafini *et al.* (1996) attributed the higher *in vivo* and *in vitro* antioxidant potency obtained for green (unfermented) tea (*Camellia sinensis)* to the presence of a higher concentration of simple hydroxyl-rich polyphenols in comparison to black (fermented) tea. In a study measuring the bleaching rate of DPPH$^{\bullet}$ by tea (*Camellia sinensis*), Manzocco *et al.* (1998) ascribed the decrease in the radical-scavenging properties of black tea compared to green tea to the enzymatic oxidation of polyphenols during black tea manufacture.

The overall relative order of antiradical efficiency of the five *Cyclopia* species to DPPH$^{\bullet}$ was: ses(U) > int(U) ≈ mac(U) ≈ ses(F) ≥ gen(U) ≥ sub(U) > gen(F) > int(F) ≥ mac(F) ≥ sub(F). The soluble solids of unfermented *C. sessiliflora* with the highest total polyphenol and flavonoid contents ($P \leq 0.05$) (Table 2) had the lowest EC_{50} value and thus the highest H-donating ability to DPPH$^{\bullet}$. Fermented *C. subternata*, followed closely by fermented *C. maculata* and fermented *C. intermedia*, showed the lowest H-donating ability to DPPH$^{\bullet}$ ($P \leq 0.05$). This is attributed to the relative low total polyphenol content of their soluble solids ($P \leq 0.05$). Fermented *C. sessiliflora*, unfermented *C. maculata* and unfermented *C. intermedia* had similar H-donating ability ($P \geq 0.05$), but were *ca.* 20% more active to DPPH$^{\bullet}$ than unfermented *C. subternata*, although the total polyphenol content of their soluble solids did not differ significantly ($P \geq 0.05$). This also suggests that the unfermented *C. subternata* contains phenolic compounds with a weaker H-donating ability.

XOD inhibitory assay of aqueous extracts from *C. intermedia*

Samples containing aqueous extracts from fermented and unfermented *C. intermedia* had a lower rate of uric acid production when compared to the control (Fig. 2). This is an indication of an inhibitory effect of both tea extract towards the XOD enzyme (Costantino *et al.*, 1992). This moderate inhibition of the rate of uric acid production may be partially due to the inhibitory effect of phenolic compounds present in the tea on the activity of XOD. For instance, naringenin and luteolin, both present in fermented *C. intermedia* (Ferreira *et al.*, 1998), respectively showed moderate (Iio *et al.*, 1985) and strong inhibition (Noro *et al.*, 1983) of the activity of XOD.

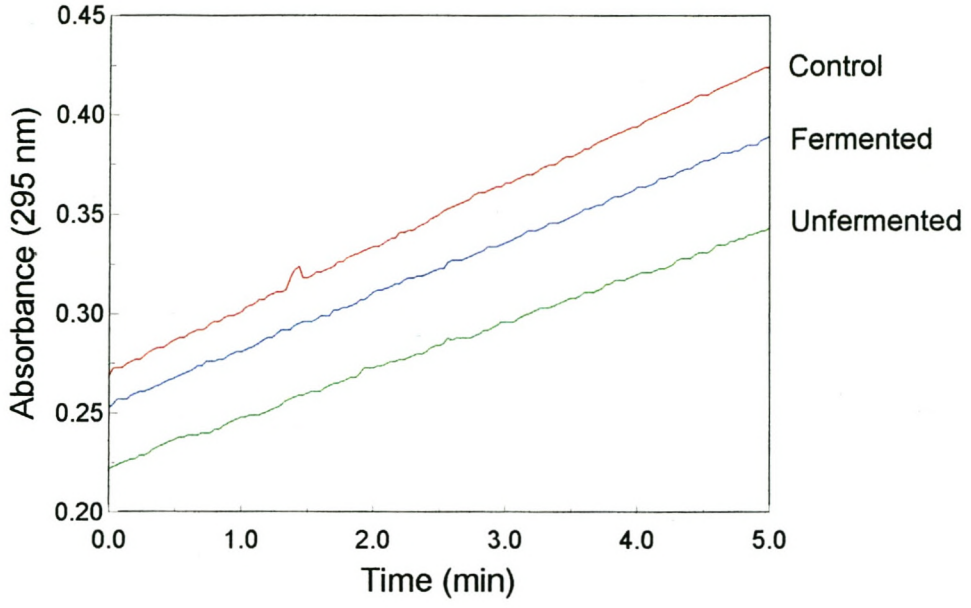

Figure 2 Inhibitory effect of aqueous extracts of fermented and unfermented *C. intermedia* towards XOD as shown by a decrease in the rate of uric acid production at 295 nm compared to a control.

The slightly stronger inhibition of XOD by unfermented *C. intermedia* (21.8%) than its fermented equivalent (12.5%) as calculated from Fig. 2, could therefore be due to a higher total polyphenol content in the unfermented material. Based on these results, the enzymatic generation of $O_2^{\bullet-}$ by the xanthine-XOD system was not used in this study, since the $O_2^{\bullet-}$ scavenging ability of extracts will be misinterpreted.

In human tissue, XOD normally oxidizes the complex molecule hypoxanthine to xanthine, after which xanthine is further oxidized to uric acid. Both $O_2^{\bullet-}$ and H_2O_2 are generated during the oxidation process which may have harmful consequences in the body (Gutteridge & Halliwell, 1994). The inhibitory effect of fermented *C. intermedia* on XOD could possibly have a positive impact on the human body in that less harmful reactive intermediates, including $O_2^{\bullet-}$ and H_2O_2, are produced. However, no *in vivo* studies have been undertaken up to date to investigate honeybush tea as antioxidant.

Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity

The *in vitro* antiradical activity of aqueous extracts prepared from fermented and unfermented *Cyclopia* species towards non-enzymatically generated $O_2^{\bullet-}$ was expressed as IC_{50} values (Table 4). The lower the IC_{50} value of a specific extract, the more effective the scavenging effect towards $O_2^{\bullet-}$, i.e. progressively less soluble solids would be necessary to scavenge a fixed $O_2^{\bullet-}$ concentration. All *Cyclopia* extracts scavenged $O_2^{\bullet-}$ by inhibiting formazan formation, however, the potency of this inhibitory action differed between species.

A typical result as shown in Fig. 3, shows formazan formation over time for aqueous extracts from fermented *C. maculata*, illustrating that less blue formazan was formed in a concentration-dependant manner with increasing soluble solids in the extracts. From the results obtained for the different samples, a log dose-inhibition plot was compiled for each dilution series of each sample, from which the IC_{50} values were calculated. The data in Fig. 4 illustrates the log dose-inhibition plot for fermented *C. maculata*.

Table 4 $O_2^{\bullet-}$ scavenging activity of aqueous extracts from different *Cyclopia* species as affected by fermentation

Species	IC_{50} [1,2]	
	Unfermented	**Fermented**
C. sessiliflora	60.8 a	97.0 b
C. genistoides	109.9 b	132.4 c
C. maculata	158.8 d	249.3 f
C. subternata	96.7 b	151.8 d
C. intermedia	170.5 de	238.2 f

[1] Each value represents the means of four replicates (n = 4). Means within the table followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[2] Expressed as µg soluble solids mL^{-1} reaction mixture required to inhibit NBT reduction by 50%.

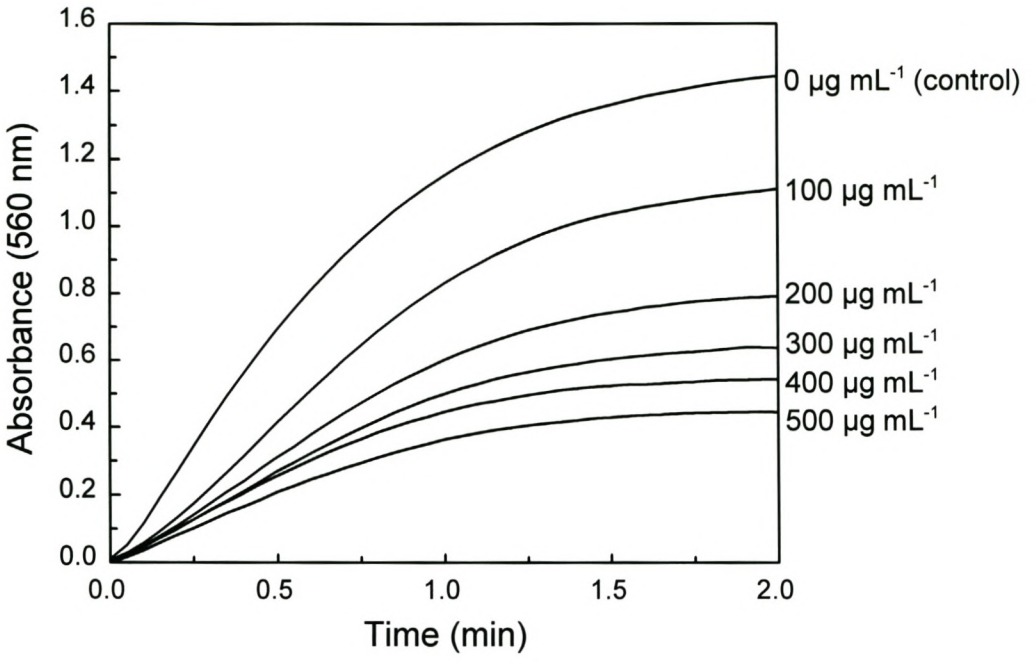

Figure 3 The influence of aqueous extracts from fermented *C. maculata* containing between 100 and 500 µg soluble solids mL^{-1} reaction mixture (blank values subtracted) on $O_2^{\bullet-}$ generated NBT reduction (λ = 560 nm), compared to a control without the inhibitor.

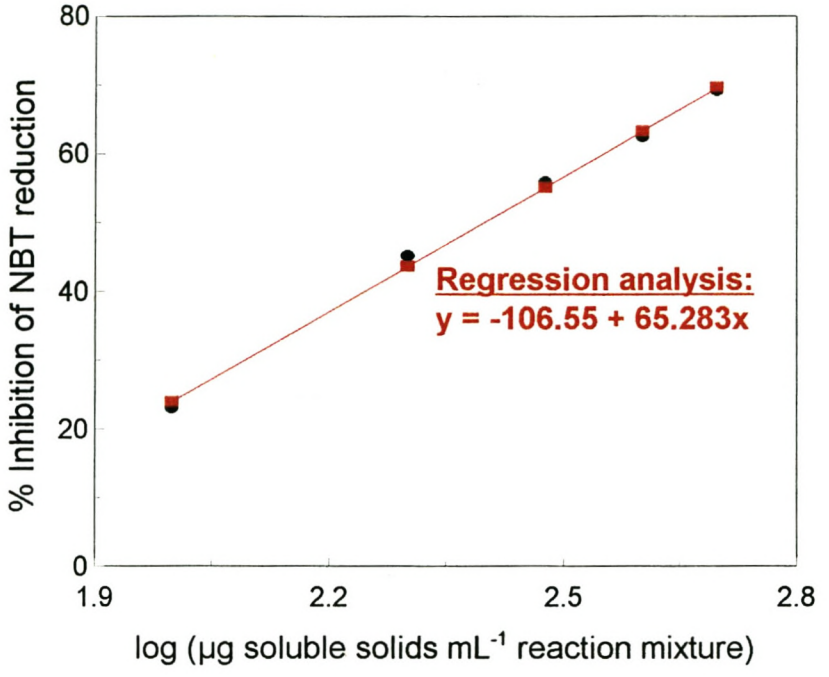

Figure 4 Log dose-inhibition plot of inhibition (%) of NBT reduction as a function of the log amount (µg) of soluble solids mL^{-1} reaction mixture of an aqueous extract from fermented *C. maculata* (IC_{50} = 250.04).

The $O_2^{\bullet-}$ scavenging activity of unfermented *Cyclopia* species decreased in the order: Ses > Sub ≈ Gen > Mac ≥ Int. The IC_{50} values varied between 60.8 and 170.5 µg soluble solids mL^{-1} reaction mixture. For fermented *Cyclopia* species with higher IC_{50} values (97.0 to 249.3 µg soluble solids mL^{-1} reaction mixture), the decreasing order was different: Ses > Gen > Sub > Int ≈ Mac. For all five *Cyclopia* species, the unfermented extracts with soluble solids containing between 23.2 and 32.8% (m/m) total polyphenols scavenged $O_2^{\bullet-}$ more effectively than their respective fermented equivalents with soluble solids containing between 12.2 and 23.3% (m/m) total polyphenols.

The overall ability of aqueous extracts from different *Cyclopia* species to scavenge $O_2^{\bullet-}$ decreased in the order: ses(U) > sub(U) ≈ ses(F) ≈ gen(U) > gen(F) > sub(F) ≈ mac(U) ≥ int(U) > int(F) ≈ mac(F). For all the species tested, the aqueous soluble solids of unfermented *C. sessiliflora*, with the highest total polyphenol and flavonoid contents (Table 2), showed the lowest IC_{50} value ($P \leq 0.05$) and, consequently, the most effective $O_2^{\bullet-}$ scavenging activity. Fermented *C. maculata* and *C. intermedia* showed the highest IC_{50} values ($P \leq 0.05$) and therefore the weakest $O_2^{\bullet-}$ scavenging activity. The soluble solids of fermented *C. subternata*, which did not differ significantly from fermented *C. maculata* and *C. intermedia*, had the lowest total polyphenol content (Table 2). Fermented *C. subternata*, on the contrary, was more effective than fermented *C. maculata* and *C. intermedia*, probably indicating the presence of phenolic compounds with stronger $O_2^{\bullet-}$ scavenging activity.

Unfermented *C. maculata* and *C. intermedia*, and fermented *C. subternata* showed the same $O_2^{\bullet-}$ scavenging activity ($P \geq 0.05$) (Table 4). This is irrespective of the soluble solids of fermented *C. subternata* containing 50% less total polyphenols than the other two extracts (Table 2), again indicating a qualitative difference in their phenolic composition. The soluble solids of unfermented *C. subternata* and fermented *C. sessiliflora*, having the same total polyphenol contents, scavenged $O_2^{\bullet-}$ to the same degree ($P \geq 0.05$). Both these extracts showed *ca.* 40% stronger $O_2^{\bullet-}$ scavenging activity than unfermented *C. maculata* and unfermented *C. intermedia*, although there was no significant difference ($P \geq 0.05$) between the total polyphenol contents. This indicates once again that specific compounds in extracts, rather than the total polyphenol contents, are important for effective $O_2^{\bullet-}$ scavenging ability.

The overall relative order of antiradical efficiency of *Cyclopia* extracts differed for the DPPH$^{\bullet}$ and $O_2^{\bullet-}$ scavenging methods. Several possible explanations may account for these differences. According to Yoshida *et al.* (1989), the antiradical power of the polyphenols is strongly influenced by the nature of the radical species (eg. DPPH$^{\bullet}$) under investigation. The restricted accessibility of the radical centre of DPPH$^{\bullet}$ to different polyphenols (Yoshida *et al.*, 1989) possibly due to the steric hindrance of the DPPH molecule (Cämmerer & Kroh, 1996) in comparison to the smaller size of $O_2^{\bullet-}$, may affect activity. The solubilities of antioxidant compounds also determine their efficiency in a particular system, for instance in an aqueous environment (eg. $O_2^{\bullet-}$ scavenging method) or in an organic solvent (eg. DPPH$^{\bullet}$ scavenging method) (Halliwell, 1990).

The $O_2^{\bullet-}$ scavenging activities of aqueous extracts from different *Cyclopia* species were also compared to that of SOD, an important biological antioxidant defence enzyme (Gutteridge & Halliwell, 1994). In mammalian cells, SOD rapidly converts $O_2^{\bullet-}$ into H_2O_2, which is in return rapidly removed by catalase (Halliwell, 1990). Superoxide dismutase mimic units were used to express the activity of tannins and flavonoids mimicking SOD by scavenging $O_2^{\bullet-}$ generated in a xanthine-XOD system (Okamura *et al.*, 1993). The $O_2^{\bullet-}$ scavenging activities of green tea extracts, compared with that of pure SOD by using electron spin resonance (ESR) spectroscopy, were expressed in terms of superoxide radical scavenging activity (SOSA) values (Ramarathnam *et al.*, 1995). In this study it was found that SOD inhibited the $O_2^{\bullet-}$-induced NBT reduction in a concentration-dependant manner, in a similar fashion as shown for fermented *C. maculata* (Fig. 3). The average IC_{50} value for SOD was 35.25 µg mL^{-1} reaction mixture (data not shown), compared to the 60.8 µg soluble solids mL^{-1} for the most effective species, unfermented *C. sessiliflora* (Table 4). Tea soluble solids are, therefore, less effective as scavengers of $O_2^{\bullet-}$ than SOD when compared on a mass basis.

Inhibition of linoleic acid peroxidation

The ability of aqueous extracts from different *Cyclopia* species to inhibit the formation of CD hydroperoxides resulting from linoleic acid peroxidation (Lingnert *et al.*,

1979) was compared to BHA and BHT. The higher the inhibition (%) of CD hydroperoxides (at t = 21 h), the higher the protective effect of the test sample towards the peroxidation of linoleic acid, and *vice versa*. The method initially gave poor reproducibility, possibly due to metal ions leaching out of reaction vials, thereby accelerating peroxidation of linoleic acid. The problem was eliminated by soaking glassware in an EDTA solution to chelate metal ions (Farag *et al.*, 1989).

The data in Fig. 5 shows the increasing absorbance (λ = 234 nm) in the control and samples (blank values already subtracted) due to the formation of CD hydroperoxides in the linoleic acid emulsion as a function of time (h). For each sample, rates of linoleic acid peroxidation (i.e. gradient) and inhibition (%) of CD hydroperoxide formation (t = 21 h) are given in Table 5.

All five *Cyclopia* species and reference compounds, BHT and BHA, inhibited linoleic acid peroxidation significantly ($P \leq 0.05$) compared to the control. The overall inhibitory effect for different *Cyclopia* species towards linoleic acid peroxidation was in decreasing order: Gen > Sub > Ses > Mac > Int(F) > Int(U) ($P \leq 0.05$). Unfermented *C. intermedia* was still relatively effective at 58.5% inhibition of CD hydroperoxide formation. BHT and BHA almost completely inhibited the formation of CD hydroperoxides with respectively 98.1 and 97.6% inhibition, which is slightly more effective than the most effective species, *C. genistoides*.

The proposed mechanism for the inhibitory effect of *Cyclopia* extracts towards linoleic acid peroxidation, a free radical process, may be attributed to the ability of their phenolic constituents to efficiently donate H thereby breaking radical chain reactions (Eriksson & Na, 1995). Duh (1998) ascribed the inhibition of linoleic acid peroxidation of burdock (a Chinese vegetable) extracts to their H-donating abilities towards free radicals. However, the present results indicate that there was no correlation between the relative order of efficiency of different *Cyclopia* extracts to donate H to $DPPH^{\bullet}$ (Table 3) and their inhibition of linoleic acid peroxidation (Table 5).

Polyphenols contain several groups or regions (eg. aromatic ring structure, the C-H skeleton of sugars, etc.) thereby providing a multiplicity of sites with a potentially hydrophobic nature (Haslam, 1996). Consequently, these groups and regions in different molecules associate and congregate together, resulting in a reduced overall surface area exposed to the aqueous phase causing a driving force towards the lipid phase (Haslam, 1996).

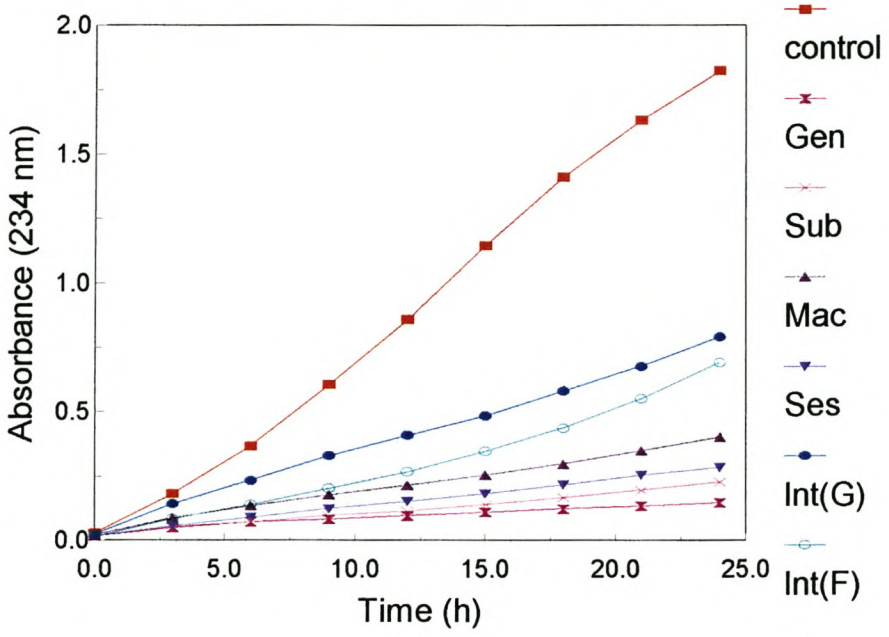

Figure 5 Efficiency of aqueous extracts from *Cyclopia* species to inhibit linoleic acid peroxidation in a linoleic acid emulsion as monitored at 3 h-intervals for 21 h at 234 nm, compared with a control. Fermentation had no significant effect ($P \geq 0.05$) on the inhibitory effect towards linoleic acid peroxidation, therefore all the tea species (except *C. intermedia*) were represented by a single line on the graph.

Table 5 The inhibition of CD hydroperoxide formation and rate of linoleic acid peroxidation in a linoleic acid emulsion for fermented and unfermented *Cyclopia* species and two synthetic antioxidants, BHA and BHT

Test material	**Inhibition (%) of CD hydroperoxides** [1,2]	**Rate ($\times 10^{-2}$) of linoleic acid peroxidation** [1,3]
BHT	98.1 a	0.01 a
BHA	97.6 a	0.06 a
C. genistoides	91.3 b	0.52 b
C. subternata	88.2 bc	0.81 bc
C. sessiliflora	84.8 c	1.06 c
C. maculata	78.7 d	1.50 d
C. intermedia - fermented	66.2 e	2.68 e
C. intermedia - unfermented	58.5 f	3.07 f
control		7.88 g

[1] Each value represents the means of three replicates (n = 3) with duplicate analysis. Means within a column followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests for inhibition (%) of CD hydroperoxides and pairwise Student's t-LSD for rate of linoleic acid peroxidation.

[2] Inhibition (%) of CD hydroperoxide formation in the reaction medium calculated after 21 h incubation.

[3] Calculated following linear regression of single lines fitted to graphs ($\Delta A/\Delta t$).

The relatively high inhibition (%) of linoleic acid peroxidation by *Cyclopia* extracts could, therefore, either indicate their efficiency at the interface of the linoleic acid droplets in the emulsion, or partitioning of the phenolic compounds into the lipid phase. The higher activity of *C. genistoides* compared to *C. intermedia* could indicate the presence of highly lipophilic (nonpolar) compounds in the former extract thereby exerting their protective effect towards linoleic acid peroxidation.

Contrary to the results of the DPPH$^{\bullet}$ and $O_2^{\bullet-}$ scavenging assays, fermentation had no significant effect ($P \geq 0.05$) on the efficiency of different species, except *C. intermedia*, towards linoleic acid peroxidation (Table 5). This non-discriminating inhibition of linoleic acid peroxidation by fermented and unfermented *Cyclopia* extracts may be explained by quantitative and qualitative differences in their polyphenols partitioning into the different phases. Honeybush tea polyphenols are oxidized during fermentation thereby becoming more non-polar, resulting in an increased affinity for the lipid phase. Thus, fermented *Cyclopia* extracts showed an increased efficiency towards inhibition of linoleic acid peroxidation, in spite of the decrease in their phenolic content with fermentation. Unfermented *Cyclopia* extracts, however, had a higher non-oxidised polyphenol content thereby increasing its polarity, resulting in the same efficiency as the fermented tea.

A similar trend was reported by Hodgson *et al.* (1999) for the inhibitory effect of green and black tea (*Camellia sinensis*) towards low-density lipoprotein (LDL) oxidation. The green tea had a higher non-oxidised flavonoid content than the black tea, but the total polyphenol concentrations did not differ significantly. Similarly, the greater solubility of epigallocatechin (EGC) - a green tea polyphenol - in the aqueous phase explains its lesser efficiency in inhibiting lipid peroxidation (Rice-Evans, 1999). The low antioxidant activity of 1,4-dihydropyridine derivatives is attributed to the hydrophilicity of the compounds and as a result, the lack of partitioning into peroxidizing micelles, regardless of the high H-donating ability to DPPH$^{\bullet}$ (Abdalla *et al.*, 1999).

Lipophilic (non-polar) antioxidants (eg. BHA and BHT) have been reported to show activity in polar lipid emulsions by being orientated in the oil-water interface (Porter *et al.*, 1989). In the present study, BHA and BHT indeed showed the most effective inhibition (> 95%) of linoleic acid peroxidation ($P \leq 0.05$) (Table 5).

Conclusions

Both species identity and fermentation affected the antiradical efficiencies, i.e. the scavenging of $DPPH^{\bullet}$ and $O_2^{\bullet-}$, of aqueous *Cyclopia* extracts to varying degrees when compared on a mass basis. This is attributed to the variation in the phenolic constituents between different species (De Nysschen *et al.*, 1996) and the declining total polyphenol content of honeybush tea extracts during fermentation (Du Toit & Joubert, 1998). Aqueous extracts from the *Cyclopia* species containing the highest total polyphenol content showed the highest antiradical potential and *vice versa*, but total polyphenol content did not correlate with relative efficiency. Fermentation did not affect the efficiency of the *Cyclopia* extracts against lipid peroxidation, nor could the efficiency of the most effective species, *C. genistoides*, be attributed to the phenolic content. Factors such as partitioning and solubility of polyphenols within the oil-water interface of the linoleic acid emulsion are therefore important considerations towards inhibition of linoleic acid peroxidation.

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Chapter 4

Antioxidant and antiradical activities of aqueous extracts, crude phenolic fractions and phenolic compounds from *Cyclopia intermedia*

Abstract

The antioxidant and antiradical potential of aqueous extracts as well as crude phenolic methanol and diethyl ether fractions prepared from fermented and unfermented material of *Cyclopia intermedia* were investigated. Phenolic compounds from fermented *C. intermedia*, including naringenin, eriodictyol, hesperetin, hesperidin, luteolin, formononetin, medicagol, flemichapparin, mangiferin, isomangiferin and 4-coumaric acid were also evaluated for their antioxidant and antiradical potential. For the purpose of evaluating test materials of this divergent nature in the same assay, comparison was executed on a mass basis. However, to elucidate structure-activity relationships of phenolic compounds, the comparison was also performed on a molar basis. The hydrogen-donating (H-donating) ability of test materials towards 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH$^{\bullet}$) was assayed using quercetin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as reference compounds. Fermentation of *C. intermedia* decreased the H-donating abilities of aqueous extracts and crude phenolic fractions. The overall decreasing order of efficiency of all test materials towards DPPH$^{\bullet}$ on a mass basis was as follows: quercetin ≈ luteolin > eriodictyol ≥ BHA ≥ mangiferin ≈ isomangiferin > BHT ≈ diethyl ether fraction (unfermented, UD) ≈ methanol fraction (unfermented, UM) > aqueous extract (unfermented, UA) > aqueous extract (fermented, FA) > methanol fraction (fermented, FM) ≈ diethyl ether fraction (fermented, FD) ≈ hesperetin > hesperidin. Formononetin, flemichapparin, medicagol, 4-coumaric acid and naringenin were ineffective towards DPPH$^{\bullet}$. The efficiency of all test materials was also investigated in the superoxide anion radical ($O_2^{\bullet-}$) scavenging assay, using

quercetin and an antioxidant enzyme, superoxide dismutase (SOD), as reference compounds. Fermentation of *C. intermedia* also decreased the ability of aqueous extracts and crude phenolic fractions to scavenge $O_2^{\bullet-}$. The most effective inhibitors of $O_2^{\bullet-}$ in decreasing order, on a mass basis, were: quercetin > SOD ≈ luteolin ≈ eriodictyol > UD > UA > UM ≈ FA > mangiferin. Crude phenolic fractions of fermented *C. intermedia* (FM and FD) and naringenin, hesperetin and hesperidin were poor scavengers of $O_2^{\bullet-}$, while formononetin, flemichapparin, medicagol, 4-coumaric acid, BHA and BHT were ineffective. The effect of all test materials on linoleic acid peroxidation was measured, on an equal mass basis, using BHA, BHT and quercetin as reference compounds. Butylated hydroxyanisole (BHA), BHT, luteolin, eriodictyol, mangiferin, isomangiferin and hesperetin effectively inhibited linoleic acid peroxidation ($P \geq 0.05$), while formononetin, flemichapparin, medicagol and naringenin were ineffective. Aqueous extracts and crude phenolic fractions from fermented and unfermented *C. intermedia*, inhibited linoleic acid peroxidation to the same degree. The aqueous extract of fermented *C. intermedia* (FA), as well as FM, UM, FD and UD, showed inhibition of linoleic acid peroxidation comparable to that of quercetin ($P \geq 0.05$).

Introduction

Cyclopia intermedia is currently the most important commercial species of the more than 20 species of honeybush tea endemic to the indigenous Cape fynbos region of South Africa (Van Wyk *et al.*, 1997). This originates from its continued use through the years and the availability of plant material for processing. This particular species is currently still harvested from wild populations in the mountainous areas of the Langkloof. In the period April 1998 to March 1999 about 50 tons of *C. intermedia* were exported to Germany, the United Kingdom and United States of America, compared to the eight tons that were distributed to local supermarkets and farm stalls. The overall demand for honeybush tea during the 1999/2000 period, is estimated at *ca.* 100 tons of which 80% was exported (N. Coetzee, Coetzee & Coetzee Distributers and D. de Villiers, Cape Natural Tea Products, Cape Town, South Africa, personal communication, 2000).

The commercial importance of *C. intermedia* prompted the investigation of the phenolic profile of the fermented material from this species in particular. Initially, the

phenolic compounds, including coumestans (eg. medicagol and flemichapparin), xanthone C-glycosides (mangiferin and isomangiferin), a variety of $C_6.C_3.C_6$-type polyphenols (flavanones, isoflavones and a flavone), a hydroxy-cinnamic acid (4-coumaric acid), and an inositol ((+)-pinitol), were isolated from this material (Ferreira *et al.*, 1998). A follow-up investigation by Kamara (1999) yielded phenolic compounds, including flavones, glycosylated flavonols, flavanones, isoflavones, and $C_6.C_1$- and $C_6.C_2$-type phenols. Several of the phenolic metabolites isolated from fermented *C. intermedia*, such as eriodictyol, hesperetin, hesperidin, naringenin, luteolin and 4-coumaric acid, showed antioxidant and antiradical activities in various *in vitro* and *in vivo* model systems (Sato *et al.*, 1992; Joyeux *et al.*, 1995; Haraguchi *et al.*, 1996; Rice-Evans *et al.*, 1996; Miyake *et al.*, 1997a,b). However, little or no information is available on the relative efficiency of xanthones and coumestans as antioxidants in different systems, and in comparison to other types of phenolic compounds such as found in fermented *C. intermedia*.

There has been an increasing interest in phenolic compounds from natural sources, as they may possess important pharmacological properties, such as antioxidant activity or inhibition of carcinogenesis (Shahidi & Naczk, 1995). Honeybush tea is a potential source of antioxidants due to the presence of phenolic compounds, in addition to its use as a refreshing herbal beverage. The synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are applied in the food industry in order to retard the oxidation of fats and oils (Chu & Hsu, 1999). Concern about possible harmful effects of these synthetic chemicals *in vivo*, has additionally prompted the investigation of the antioxidative properties of naturally occurring compounds (Aruoma *et al.*, 1990; Kim *et al.*, 1994; Yen & Duh, 1994), such as those from honeybush tea.

The aim of this investigation was to establish the relative efficiency of phenolic compounds from fermented *C. intermedia* as antioxidants and radical scavengers. Synthetic antioxidants and known radical scavengers were used for comparative purposes. Crude phenolic fractions, prepared from unfermented and fermented *C. intermedia*, were tested for antioxidant and antiradical properties and compared with aqueous extracts from *C. intermedia*. *In vitro* test systems, including the hydrogen-donating (H-donating) ability towards the 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$),

the superoxide anion radical ($O_2^{\bullet-}$) scavenging method, and the inhibition of linoleic acid peroxidation, were used to assess antioxidant and antiradical properties.

Materials and methods

Chemicals

A detailed description of all chemicals used was given in Chapter 3. For the preparation of crude phenolic fractions, solvents (chloroform, acetone, methanol and diethyl ether) of analytical reagent (AR) grade were used (BDH, Poole, England). For high performance liquid chromatography (HPLC) analysis, methanol (HPLC grade) was obtained from BDH (Poole, England) and formic acid (AR) from Merck (Darmstadt, Germany).

Deionised water refers to water sequentially treated with carbon, reverse osmosis (RO) and a mixed-bed deioniser to obtain a conductivity of 0.1 µs cm^{-1} using a Modulab Water Purification System (Continental Water Systems Corporation, Texas, USA).

Apparatus

All spectrophotometric measurements were performed in a 1 cm path length quartz cell (except when noted otherwise) using a Beckman DU-65 spectrophotometer equipped with ultraviolet (UV) and visible (VIS) sources. Data from time assays were recorded with Beckman Data Capture Software. High performance liquid chromatography (HPLC) analysis was performed on a Varian 5000 (Vista series) liquid chromatograph using a variable UV absorption detector at a wavelength of 320 nm. Peak areas were measured using a Hewlett Packard HP 3390A reporting integrator.

Phenolic compounds of fermented *C. intermedia*

Isomangiferin, flemichapparin and medicagol, all isolated from dried, pulverized material of fermented *C. intermedia* (*ex* Langkloof), were kindly supplied by Prof. Daneel Ferreira, formerly of the Department of Chemistry, University of the Orange Free State

(UOFS), Bloemfontein, South Africa. Formononetin and hesperidin were purchased from Extrasynthése (France) and L. Light & Co. Ltd. (Colnbrook, England), respectively. Naringenin, hesperetin, mangiferin and 4-coumaric acid were obtained from Sigma Chemical Co. (St Louis, USA). Quercetin, as reference compound, was from Merck (Darmstadt, Germany), while eriodictyol and luteolin were obtained from Carl Roth GmbH (Karlsruhe, Germany). All phenolic compounds were dissolved in dimethyl sulphoxide (DMSO) for testing, due to poor solubility of some compounds in deionised water, methanol and ethanol.

Harvesting and processing of plant material

The harvesting and processing of four separate bundles of plant material (representing four replicates) from *C. intermedia* (*ex* Langkloof) used for the preparation of the aqueous extracts, were described in Chapter 3. *Cyclopia intermedia* (*ex* Langkloof) (*ca.* 21 kg) used for the preparation of crude phenolic fractions, was harvested in January 1998 from natural-growing populations in the mountainous area of Vallei Vrugteplase, Louterwater, Langkloof, Eastern Cape Province, South Africa. Excessive woody material was removed before cutting, whereafter fermented and unfermented material were prepared as described in the previous Chapter. The dried, sieved material ($\leq$ 2 mm) of fermented and unfermented *C. intermedia* was pulverized using a laboratory hammermill (Serial no 401, Scientific RSA).

Preparation of aqueous extracts and crude phenolic fractions

Aqueous extracts prepared from unfermented and fermented *C. intermedia* (described in Chapter 3), will be referred to as UA and FA, respectively, in the present Chapter. The pulverized unfermented and fermented plant material of *C. intermedia* were extracted and fractionated according to a slightly modified version of the method used by Ferreira *et al.* (1998). The procedure is schematically illustrated in Fig. 1. All extractions were conducted at room temperature (*ca.* 25°C) with constant agitation in glass beakers covered with aluminium foil to prevent excessive evaporation of the solvent.

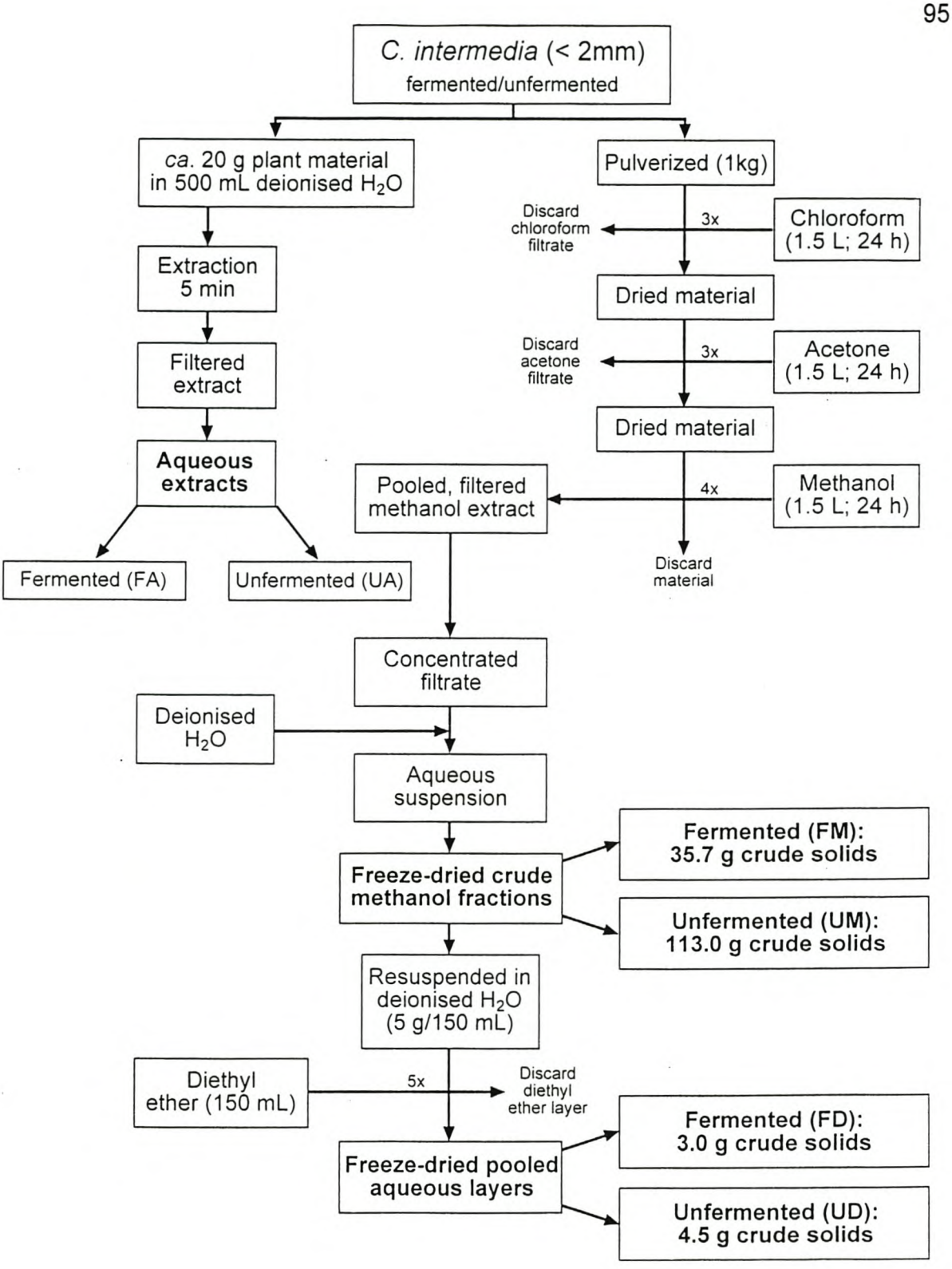

Figure 1 Preparation of aqueous extracts (FA and UA), and crude methanol (FM and UM) and diethyl ether (FD and UD) fractions from fermented and unfermented *C. intermedia*

Plant material (*ca.* 1 kg) was successively extracted with chloroform (3 x 1.5 L; 24 h each) to remove chlorophyll whereafter the material was allowed to dry (*ca.* 25°C) overnight. The process was continued by extracting the dried plant material successively with acetone (3 x 1.5 L; 24 h each). The material was again allowed to dry (*ca.* 25°C) overnight, followed by successive extraction with methanol (4 x 1.5 L; 24 h each). The mixture was filtered under vacuum through a Büchner filter containing Whatman no. 54 filter paper. Successive methanol extracts were combined and the methanol evaporated under vacuum at *ca.* 36°C using a Büchi Rotavapor until a "syrup" was obtained. Deionised water was added to the residue and evaporation continued to remove the remainder of the organic solvent from the extracts. The concentrated extracts were thoroughly rinsed from the round-bottom flasks into beakers using deionised water. Extracts were properly mixed to form homogenous solutions, decanted into plastic petri dishes and subsequently freeze-dried using an Atlas pilot-scale freeze-drier (Danmark model, Copenhagen, Denmark). The freeze-dried material was stored under vacuum in a desiccator until further testing. The fractions, FM and UM, yielded crude solids of 35.7 g and 113.0 g, respectively. A portion (*ca.* 5 g) of each of the freeze-dried FM and UM, suspended in deionised water (150 mL), was subsequently extracted with diethyl ether (5 x 150 mL) in separating funnels. The aqueous layers of both fermented and unfermented honeybush were freeze-dried to yield, respectively, 3.0 and 4.5 g of crude solids.

HPLC analysis of crude methanol fractions

Stock solutions of 1 mg crude solids mL^{-1} methanol were prepared of FM and UM, respectively, for qualitative determination of the mangiferin/isomangiferin content using HPLC. Of each fraction, a 10 µL-volume was injected onto a Phenomenex Luna column (C_{18} reverse-phase, 3 µm particle size, 150 mm length x 4.6 mm internal diameter) maintained at 38°C. Separations were performed by gradient elution using a mobile phase of methanol (solvent A)-formic acid (30:70, v/v, initial concentration) with the following solvent gradient: 0 to 20 min: 30 to 100% A; 20 to 30 min: 100% A and 30 to 50 min: 100 to 30% A. A flow rate of 1 mL min^{-1} was used.

Determination of total polyphenol contents

The total polyphenol contents of aqueous extracts and crude phenolic fractions were determined as described in Chapter 3. Results were expressed in terms of g gallic acid equivalents (GAE) 100 g^{-1} soluble solids. Results of the total polyphenol contents of aqueous extracts represent four replicates with duplicate analysis, but for crude phenolic fractions the total polyphenol contents of two replicates were determined in duplicate.

Hydrogen-donating ability ($DPPH^{\bullet}$ scavenging activity)

The H-donating ability of the test materials towards the stable free radical $DPPH^{\bullet}$ was quantified according to Brand-Williams *et al.* (1995) with modifications as described in Chapter 3. The two synthetic antioxidants, butylated hydroxyanisole (BHA) and BHT, and the most ubiquitous food flavonoid, quercetin (Formica & Regelson, 1995), were used as reference compounds. The H-donating abilities of all test materials were compared on a mass basis. In addition, the activity of phenolic compounds was compared on a molar basis to elucidate structure-activity relationships. Stock solutions (4 g L^{-1}) of FM, UM, FD and UD were prepared in methanol. Stock solutions of phenolic compounds and synthetic antioxidants, BHA and BHT, giving concentrations between 0.32 g L^{-1} and 3.66 g L^{-1}, were prepared in DMSO. Stock solutions were further diluted with methanol or DMSO to give a dilution range scavenging 20 to 80% of $DPPH^{\bullet}$.

The range of dilutions from each stock solution (50 µL) was incubated with a $DPPH^{\bullet}$ solution (2 mL, initial concentration of 3 x 10^{-5} mol L^{-1}) for 2 h in the dark to reach steady state conditions before measuring the absorbance at 515 nm. Controls were prepared by addition of the $DPPH^{\bullet}$ solution to either DMSO (for testing phenolic compounds), methanol (for testing crude phenolic fractions) or deionised water (for testing aqueous extracts). The EC_{50} values, i.e. µg antioxidant 50 μL^{-1} solution (or µM antioxidant in the reaction mixture) necessary to scavenge the initial [$DPPH^{\bullet}$] in the reaction mixture with 50%, were normalised to compensate for small variations in the initial [$DPPH^{\bullet}$].

Superoxide anion radical ($O_2^{\bullet-}$) scavenging ability

The $O_2^{\bullet-}$ scavenging method was carried out as described in Chapter 3 according to the non-enzymatic method of Robak & Gryglewski (1988). The potential of the test materials to scavenge $O_2^{\bullet-}$ was compared with superoxide dismutase (SOD), BHA, BHT and quercetin on a mass basis. The activity of phenolic compounds was also compared on a molar basis to compensate for reduced activity due to structural differences, i.e. glycosylation. Stock solutions of 4 and 8 g L^{-1} in methanol were prepared for crude unfermented (UM, UD) and fermented (FM, FD) fractions, respectively. Stock solutions of the honeybush polyphenols, BHA and BHT, ranging between 0.34 g L^{-1} and 45.50 g L^{-1}, were prepared in DMSO. The SOD stock solution (0.06 g L^{-1}) was prepared fresh daily using chilled 0.1 M sodium phosphate buffer (pH 7.4). Stock solutions were diluted with methanol, DMSO or buffer, to give within 20 to 80% inhibition of nitroblue tetrazolium (NBT) reduction.

The incubation mixture used for testing FM, UM, FD, UD and SOD was similar to that prepared for testing the aqueous extracts (Chapter 3). However, for the test samples dissolved in DMSO, $O_2^{\bullet-}$ was generated in a reaction mixture (2 mL) containing 78 µM β-nicotinamide adenine dinucleotide (NADH), 10 µM phenazine methosulphate (PMS) and 25 µM NBT (Robak & Gryglewski, 1988). In this case, the 500 µL of buffer in the control was replaced by an equivalent volume of DMSO. The reaction was followed over 8 min at 560 nm contrasting to the 2 min reaction time used for aqueous tea extracts. All assays were carried out in triplicate.

Inhibition of linoleic acid peroxidation

The inhibition of linoleic acid peroxidation by the test samples was determined according to the method of Lingnert *et al.* (1979). All phenolic compounds, crude phenolic fractions, quercetin, BHA and BHT, were evaluated on an equal mass (m/v) basis. In addition, phenolic compounds were compared on a molar basis to elucidate structure-activity relationships. Stock solutions of 0.20 g L^{-1} were prepared for all test materials giving a final concentration of 9.50 mg L^{-1} in the reaction mixture. For phenolic compounds dissolved in DMSO, and FM, UM, FD and UD dissolved in methanol,

controls were prepared by mixing 200 µL of DMSO or methanol instead of the sodium phosphate buffer (0.1 M; pH 7.4) with 4 mL of 10 mM linoleic acid emulsion. Controls and samples were incubated at 40°C for 24 h and conjugated diene (CD) formation measured spectrophotometrically (λ = 234 nm) at 3 h-time intervals.

Statistical analysis

Analysis of variance (ANOVA) (SAS Release version 6.12) was used to determine whether test samples differed in their ability to act as antioxidants. Pairwise Student's t-tests were used to compare means when an effect was significant ($P \leq 0.05$).

Results and discussion

Total polyphenol content

The total polyphenol content of the soluble solids of aqueous extracts (FA and UA) and crude phenolic fractions (FM, UM, FD and UD) from unfermented and fermented *C. intermedia* is summarised in Table 1. The data will be used to explain certain trends as observed with the different test systems discussed in this Chapter. The total polyphenol content of the soluble solids of UA is almost twice that of the fermented material, respectively, 24.2 and 13.8 g GAE 100 g^{-1} soluble solids ($P \leq 0.05$). Similarly, the crude methanol and diethyl ether fractions prepared from unfermented material (UM and UD) have a much higher total polyphenol content than the fermented equivalents (FM and FD). This is in agreement with the findings of Du Toit (1996) showing a decrease in the total polyphenol content of extracted soluble solids of *C. intermedia* with increasing fermentation time. During fermentation, oxidation of phenolic compounds and polymerization of monomeric polyphenols to yield insoluble polymers are believed to take place (Du Toit & Joubert, 1999). The Folin Ciocalteu assay in fact measures all compounds that are readily oxidizable (i.e. with available OH-groups) under the reaction conditions (Singleton *et al.*, 1999); therefore, a reduction in the number of OH-groups due to either polymerisation or formation of insoluble compounds would explain the lower values of the fermented material.

Table 1 Total polyphenol content of the soluble solids of aqueous extracts (FA and UA) and crude methanol (FM and UM) and diethyl ether (FD and UD) fractions from fermented and unfermented *C. intermedia*

Test material	**Total polyphenols per 100 g solids** [1,2]	
	Unfermented	**Fermented**
Aqueous extract	24.2 c	13.8 b
Methanol fraction	33.7 d	10.0 a
Diethyl ether fraction	33.0 d	8.3 a

[1] For aqueous extracts, each value represents the means of four replicates (n = 4) with duplicate analysis, but for fractions, two replicates (n = 2) were analysed in duplicate. Means followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[2] Concentration expressed in terms of g GAE 100 g^{-1} soluble solids.

In addition, fermentation of *C. intermedia* resulted in a pronounced decrease in the mangiferin/isomangiferin content when FM was compared to UM as indicated by HPLC analysis (Fig. 2). Both compounds eluted at similar retention times due to structural similarity and it is thus unknown in what quantities these compounds were present. The susceptibility of mangiferin and isomangiferin to oxidation during the fermentation process results in less OH-groups that are available to react with the Folin Ciocalteu reagent to contribute to blue colour formation.

The total polyphenol content of the aqueous extract of unfermented *C. intermedia* increased by *ca.* 28% during selective extraction with methanol (Table 1). This is attributed to the removal of polymeric compounds with low reactivity towards the Folin-Ciocalteu reagent and/or concentration of compounds with increased reactivity. On the contrary, a decrease of *ca.* 28% was observed in the total polyphenol content of the aqueous soluble solids of fermented *C. intermedia* with methanol fractionation. However, for both unfermented and fermented *C. intermedia*, respectively, the total polyphenol content of the crude methanol and diethyl ether fractions did not differ significantly ($P \geq 0.05$).

Hydrogen-donating ability (DPPH$^{\bullet}$ scavenging activity)

Test materials, including FA, UA, FM, UM, FD and UD, several phenolic compounds from fermented *C. intermedia*, the reference compound quercetin, and synthetic antioxidants, BHA and BHT, scavenged DPPH$^{\bullet}$ with varying degrees of efficiency (Table 2). The overall relative decreasing activity of the test samples (on a mass basis) was in the order: quercetin $\approx$ luteolin > eriodictyol $\geq$ BHA $\geq$ mangiferin $\approx$ isomangiferin > BHT $\approx$ UD $\approx$ UM > UA > FA > FM $\approx$ FD $\approx$ hesperetin > hesperidin. Naringenin, formononetin, flemichapparin, medicagol, and 4-coumaric acid were ineffective.

The higher total polyphenol contents of the aqueous extracts and crude phenolic fractions from unfermented *C. intermedia* in comparison with the fermented equivalents (Table 1) could explain the more efficient scavenging of DPPH$^{\bullet}$ and thus lower EC_{50} values (Table 2). Both UM and UD, containing the highest total polyphenol contents, were the most effective donors of H (lowest EC_{50} values) ($P \leq 0.05$).

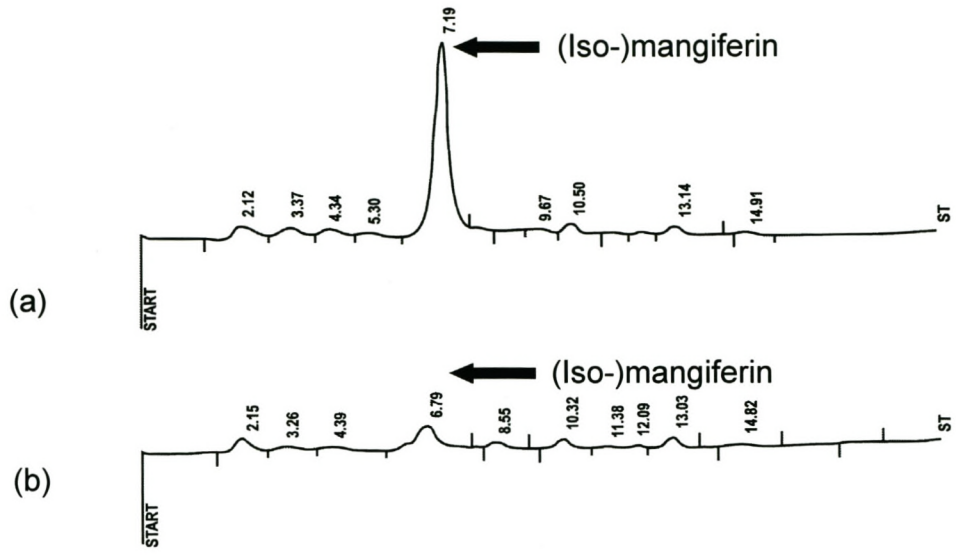

Figure 2 HPLC chromatograms of (a) UM and (b) FM, illustrating a pronounced decrease in the mangiferin/isomangiferin content of FM due to the fermentation of the material of *C. intermedia*.

Table 2 The H-donating ability towards DPPH$^{\bullet}$ and $O_2^{\bullet-}$ scavenging activity of aqueous extracts, crude phenolic fractions and compounds from *C. intermedia* in comparison with reference compounds SOD and quercetin, and the synthetic antioxidants, BHA and BHT

Test material	EC_{50} [1,2]	IC_{50} [3,4]
BHA	2.88 bc (7.77)	ineffective
BHT	6.16 d (13.61)	ineffective
SOD	–	35.85 b
Quercetin	1.77 a (2.54)	16.77 a (49.57)
Luteolin [5]	2.03 a (3.46)	39.31 b (137.33)
Eriodictyol	2.51 b (4.23)	39.53 b (137.13)
Mangiferin	3.36 c (3.88)	331.97 f (786.10)
Isomangiferin	3.38 c (3.91)	insufficient quantity
Naringenin	ineffective	515.35 g (1892.58)
Hesperetin	43.25 g (69.80)	880.88 h (2913.93)
Hesperidin	154.47 h (123.40)	1 209.87 i (1981.54)
Formononetin	ineffective	ineffective
Flemichapparin	ineffective	ineffective
Medicagol	ineffective	ineffective
4-Coumaric acid	ineffective	ineffective
FM [6]	40.26 g	> 2 000.00 k
FD	40.98 g	1 490.92 j
UM	6.85 d	226.05 e
UD	6.81 d	141.69 c
FA [7]	25.35 f	238.21 e
UA	10.46 e	170.50 d

1 Expressed as µg antioxidant 50 μL^{-1} sample (or in brackets the µM in the reaction mixture) to reduce the initial DPPH$^{\bullet}$ [3.04 x 10^{-5} mol/L] in the reaction mixture by 50%.

2 Each value represents the means of three replicates (n = 3) with duplicate analysis. Means within a column followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

3 Expressed as µg antioxidant mL^{-1} (or in brackets the µM) required in the reaction mixture to inhibit NBT reduction by $O_2^{\bullet-}$ with 50%.

4 Each value represents the means of three replicates (n = 3). Means within a column followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

5 Phenolic compounds of fermented *C. intermedia*.

6 Crude phenolic fractions of fermented and unfermented *C. intermedia*.

7 Extraction of *ca.* 20 g tea leaves with 500 mL of freshly boiled deionised water for 5 min.

Crude phenolic fractions of fermented *C. intermedia* (FM and FD) with the lowest total polyphenol contents were the least effective scavengers of DPPH$^{\bullet}$ (highest EC_{50} values of ~ 40) ($P \leq 0.05$). This is attributed to the decrease in the mangiferin/isomangiferin content of FM compared with UM, as illustrated using HPLC analysis (Fig. 2). In addition, FD consists of only *ca.* 8.6% mangiferin/isomangiferin, with (+)-pinitol comprising the remainder of the fraction (Ferreira *et al.*, 1998). The non-phenolic character of pinitol (Singleton *et al.*, 1999) could explain the weak activity of FD. Thus, the potent H-donating ability of UM and UD (average EC_{50} = 6.83) compared with the less effective FM and FD (average EC_{50} = 40.62) is attributed to the presence of (iso-)mangiferin (average EC_{50} = 3.37), which is highly effective in scavenging DPPH$^{\bullet}$ (Table 2).

Aqueous extracts and crude phenolic fractions of unfermented *C. intermedia* were *ca.* 2.4 and 5.9 times more effective in donating H to DPPH$^{\bullet}$ than their respective fermented equivalents. The overall decrease in the total polyphenol content during the fermentation process, resulting from possible oxidation of specific potent phenolic compounds and polimerization of smaller monomeric polyphenols to form insoluble compounds (Du Toit & Joubert, 1999), could account for the reduced activity of the fermented material.

For both fermented and unfermented *C. intermedia* there was no significant difference between the H-donating abilities of their crude methanol and diethyl ether fractions ($P \geq 0.05$). Since the total polyphenol content of these fractions also did not differ significantly, qualitative differences in phenolic composition did not affect H-donating ability. Crude phenolic fractions (unfermented) contain *ca.* 1.4 times more total polyphenols than the aqueous extract of unfermented *C. intermedia*, resulting in *ca.* 1.5 times stronger H-donating ability than the latter extract. Crude phenolic fractions (fermented) showed *ca.* 1.6 times less activity than the aqueous extract of fermented *C. intermedia* as it contains *ca.* 1.5 times less total polyphenols. The H-donating ability of fermented *C. intermedia* therefore decreased with selective extraction, while an improvement in the H-donating ability was observed for the unfermented material.

Of the 11 phenolic compounds from fermented *C. intermedia* that were tested, luteolin, eriodictyol, mangiferin, isomangiferin, hesperetin and hesperidin scavenged DPPH$^{\bullet}$ with varying degrees of efficiency (Table 2). Comparison on a mass basis

showed that the most effective honeybush phenolic compound ($P \leq 0.05$), luteolin (EC_{50} = 2.03), and the reference compound quercetin (EC_{50} = 1.77) had the same activity ($P \geq 0.05$). Other effective scavengers of DPPH$^{\cdot}$ were, in decreasing order of efficiency, eriodictyol (EC_{50} = 2.51), mangiferin (EC_{50} = 3.36) and isomangiferin (EC_{50} = 3.38). The xanthones, mangiferin and isomangiferin were only *ca.* 1.7 times weaker in activity than luteolin. The flavanones hesperetin (EC_{50} = 43.25) and hesperidin (EC_{50} = 154.47) were, respectively, *ca.* 17 and 62 times weaker H-donators than eriodictyol, another flavanone, when compared on a mass basis. The synthetic antioxidant, BHA (EC_{50} = 2.88), was less effective towards DPPH$^{\cdot}$ than luteolin, but did not differ significantly ($P \geq 0.05$) from eriodictyol, mangiferin and isomangiferin. Butylated hydroxytoluene (BHT) (EC_{50} = 6.16) was less effective than luteolin, eriodictyol, mangiferin and isomangiferin, but it was comparable with UM and UD (average EC_{50} = 6.83).

The criteria for effective radical scavenging (Fig. 7, Chapter 2), as derived from comparison of compounds on a molar basis (Bors *et al.*, 1990), could in broad terms elucidate the structure-activity relationship as observed with the phenolic compounds from fermented *C. intermedia*. Comparison of phenolic compounds on a molar basis (Table 2) alters the relative order of efficiency. The reference compound quercetin, exhibiting the best H-donating ability, was slightly more effective than luteolin and even more effective than the xanthones, mangiferin and isomangiferin. The latter two compounds, both with the same activity ($P \geq 0.05$), were more effective than eriodictyol ($P \leq 0.05$) when the sugar moiety in their structures are taken into consideration on a molar basis. Comparison on a molar basis versus a mass basis did not change the activity of hesperetin in relation to eriodictyol. However, hesperidin was only *ca.* 29 times as effective as eriodictyol when the sugar moiety was taken into consideration. Furthermore, the H-donating ability of BHA was only 50% as effective as mangiferin and isomangiferin when compared on a molar basis.

The order of antiradical activity obtained for different phenolic compounds could also be influenced by their accessibility of the radical centre of DPPH$^{\cdot}$ (Yoshida *et al.*, 1989). The strongest H-donating ability towards DPPH$^{\cdot}$ ($P \leq 0.05$), obtained for luteolin and quercetin, may be explained by the presence of the 2,3-double bond in conjunction with the 3',4'-dihydroxy configuration in the B-ring, both important criteria for effective radical scavenging (Bors *et al.*, 1990). The absence of the 3-OH in the C-ring of luteolin

compared with quercetin did not significantly effect its H-donating ability towards DPPH$^{\bullet}$. This is in agreement with data reported by Von Gadow *et al.* (1997), which showed that quercetin (93.3%) was slightly more effective ($P \leq 0.05$) as scavenger of DPPH$^{\bullet}$ than luteolin (90.9%) when compared on a molar basis. Joyeux *et al.* (1995) showed 59% decolouration of a methanolic solution of DPPH$^{\bullet}$ (10^{-4} M) with addition of an equal molar concentration of luteolin dissolved in DMSO.

The slightly weaker H-donating ability of eriodictyol towards DPPH$^{\bullet}$ compared with luteolin, both compounds with an equal number of phenolic hydroxyl groups, is attributed to the absence of the 2,3-double bond in the structure of eriodictyol. According to Rice-Evans *et al.* (1996), flavanones having a saturated heterocyclic C-ring in contrast to the flavones and flavonols, have a lower H-donating ability as tested with 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfic acid radical cation ($ABTS^{\bullet+}$), due to the lack of conjugation between the A- and C-rings. The substitution of the *p*-OH group in the B-ring of hesperetin by a methoxy group decreased its H-donating ability *ca.* 17-fold in comparison with that of eriodictyol, either on a mass or molar basis. This illustrates the importance of the presence of the 3',4'-dihydroxy configuration in the B-ring for effective radical scavenging. The methoxy group on the B-ring of both hesperetin and its rhamnoside, hesperidin, is accountable for the loss of activity.

The presence of rutinosyl in position 7 of the A-ring constitutes *ca.* 50% of the molecular mass of hesperidin. Thus, hesperidin (EC_{50} = 154.47) compared with hesperetin (EC_{50} = 43.25) on a mass basis, possesses only 50% of the functional groups with potential to donate H to DPPH$^{\bullet}$, thereby decreasing the H-donating ability of hesperidin to only *ca.* 28% of that of hesperetin. However, comparison on a molar basis, shows that hesperidin constitutes *ca.* 43% of the activity of hesperetin, as more functional groups are available to donate H to DPPH$^{\bullet}$. The absence of the 3',4'-dihydroxy configuration (B-ring) and the presence of 7-*O*-rutinosyl (A-ring), contribute to the poor H-donating ability of the flavanone hesperidin, when compared with another flavanone eriodictyol, either on a mass or molar basis. According to Ratty & Das (1988), glycosylation of flavonoids reduces their activity in comparison with their corresponding aglycones. Von Gadow *et al.* (1997) also showed that the presence of a glycoside resulted in a decrease in H-donating ability, since quercetin > isoquercetrin > rutin, when compared on an equal molar concentration. In another study using electron spin resonance (ESR) spectroscopy, Gardner *et al.* (1999) showed that the H-donating

ability of glycosides (rutin and myricitrin) with their 3-OH group substituted by a sugar moiety, was decreased between 10 and 15% compared with their respective aglycones (quercetin and myricetin) at an equal molar concentration of 0.2 mM.

The xanthones mangiferin (EC_{50} = 3.36) and isomangiferin (EC_{50} = 3.38) were both very effective ($P \geq 0.05$), especially considering the configuration of 2-β-D-gluco-pyranosyl either at position 2 or 4 in their otherwise identical structures. The catecholic (6,7-dihydroxy-) structure in mangiferin is more susceptible to oxidation than the *meta*-di-substituted (1,3-dihydroxy-) ring, as a radical formed at 6-OH may be delocalized to 7-OH and a radical formed at 7-OH may be delocalized to 6-OH and the heterocyclic oxygen. In addition, radicals formed at the 1-OH, 3-OH and 6-OH positions in the xanthone structure may also be delocalized to the carbonyl group (D. Ferreira, National Centre for Natural Product Research, University of Mississippi, United States of America, personal communication, 1999). These features contribute to the effective H-donating ability of mangiferin and isomangiferin towards DPPH$^{\bullet}$. Sato *et al.* (1992) showed that mangiferin causes rapid decolorization of a 0.1 mM DPPH$^{\bullet}$-solution when added at a final concentration of 50 mM in DMSO.

The flavanone naringenin with a similar structure to eriodictyol, except for the presence of a single 4'-OH group in the B-ring, showed no activity towards DPPH$^{\bullet}$ regardless of the presence of the 5-OH group (A-ring). This emphasised the importance of the 3',4'-dihydroxy configuration. Another explanation may be DMSO affecting the chemical transfer of protons in prominent positions of its flavanone structure (Grouiller & Pacheco, 1967). Rice-Evans *et al.* (1996) illustrated that the scavenging activity of naringenin, hesperetin and hesperidin towards ABTS$^{\bullet+}$ was, respectively, *ca.* 71, 66 and 51% of the activity of luteolin when compared on an equal molar basis of 1 mM. In addition to naringenin, the isoflavone formononetin, the coumestans flemichapparin and medicagol, and the hydroxy-cinnamic acid, 4-coumaric acid, did not scavenge DPPH$^{\bullet}$. Their structural arrangements do not conform with the criteria for effective radical scavenging by polyphenols. The absence of the 3',4'-dihydroxy (catechol) configuration in the B-ring, the 2,3-double bond in conjugation with a 4-oxo function in the C-ring, and both 3- and 5-OH groups (Bors *et al.*, 1990) resulted in their poor performance. The poor reaction of 4-coumaric acid with DPPH$^{\bullet}$ was also shown by Brand-Williams *et al.* (1995), Von Gadow *et al.* (1997) and Goupy *et al.* (1999).

Superoxide anion radical ($O_2^{\bullet-}$) scavenging ability

The efficiency of FA, UA, FM, UM, FD and UD, phenolic compounds of fermented *C. intermedia*, and the reference compounds, quercetin and SOD, to scavenge $O_2^{\bullet-}$ differed extensively (Table 2). The overall relative order of the test samples (on a mass basis) to scavenge $O_2^{\bullet-}$ was as follows: quercetin > SOD ≈ luteolin ≈ eriodictyol > UD > UA > UM ≈ FA > mangiferin > naringenin > hesperetin > hesperidin > FD > FM. Formononetin, flemichapparin, medicagol, 4-coumaric acid, and the synthetic antioxidants, BHA and BHT, were inefficient.

Similar to the results obtained for the H-donating ability, fermentation resulted in a decrease in the $O_2^{\bullet-}$ scavenging activities (higher IC_{50} values) of both the aqueous extracts and crude phenolic fractions (Table 2). For aqueous extracts of the unfermented material with 1.8 times the total polyphenol content of their fermented equivalents, the $O_2^{\bullet-}$ scavenging activity was *ca.* 1.4 times stronger. However, for crude phenolic fractions the contrast between the $O_2^{\bullet-}$ scavenging activities of fermented and unfermented material were more extreme. For crude methanol and diethyl ether fractions, the unfermented material was, respectively, *ca.* 9 - 10 times more effective as $O_2^{\bullet-}$scavengers than their fermented equivalents, although their total polyphenol content was only 3 - 4 times more than that of the fermented material.

The $O_2^{\bullet-}$scavenging activities of the aqueous extracts and crude phenolic fractions from *C. intermedia* (Table 2) in relation to their total polyphenol contents (Table 1), however, was not as distinct as was the case with their H-donating abilities. For both fermented and unfermented *C. intermedia* the $O_2^{\bullet-}$ scavenging activities of the aqueous extracts decreased with selective methanol extraction, but again increased during fractionation of the methanol fractions with diethyl ether. The aqueous extract of fermented *C. intermedia* (IC_{50} = 238.21) was more than 8.4 times more effective than FM (IC_{50} > 2000), while it contained *ca.* 1.4 times more total polyphenols than the latter fraction. This indicates that the aqueous extract of fermented *C. intermedia* contains polyphenols with a strong efficiency to scavenge $O_2^{\bullet-}$, compared with less potent polyphenols in FM. Fractionation of the fermented methanol fraction with diethyl ether increased the $O_2^{\bullet-}$ scavenging activity (IC_{50} = 1490.92) by more than 1.3 times, regardless that the two fractions had more or less the same polyphenol content

($P \geq 0.05$) (Table 1). Once again, this indicates the presence of polyphenols with a stronger $O_2^{\bullet-}$ scavenging potential in FD than in FM.

The aqueous extract of unfermented *C. intermedia* (IC_{50} = 170.50) was *ca.* 1.3 times more effective than the unfermented methanol fraction (IC_{50} = 226.05). In this case the higher phenolic content (*ca.* 1.4 times) could explain the increase in activity. Similar to the fermented fractions, UD (IC_{50} = 141.69) was more effective than the methanol fraction, and even more potent than the aqueous extract of unfermented *C. intermedia* (Table 2). Once again, both the methanol and diethyl ether fractions had the same total polyphenol contents ($P \geq 0.05$) (Table 1), indicating qualitative differences in the phenolic composition of these fractions.

Luteolin, eriodictyol, mangiferin, naringenin, hesperetin and hesperidin scavenged $O_2^{\bullet-}$ with varying degrees of efficiency (Table 2). Except for the reference compound quercetin (IC_{50} = 16.77), the strongest activity on a mass basis was obtained for luteolin (IC_{50} = 39.31) and eriodictyol (IC_{50} = 39.53) which were comparable ($P \geq 0.05$) with that of the antioxidant enzyme, SOD (IC_{50} = 35.85). Naringenin (IC_{50} = 515.35), hesperetin (IC_{50} = 880.88) and hesperidin (IC_{50} = 1209.87) showed, respectively, *ca.* 13, 22 and 31 times weaker $O_2^{\bullet-}$ scavenging activity than eriodictyol on a mass basis. The xanthone mangiferin (IC_{50} = 331.97) was *ca.* 8 times weaker as $O_2^{\bullet-}$ scavenger than eriodictyol, but *ca.* 1.6 times more effective than naringenin. Isomangiferin was not tested due to an insufficient quantity available. The respective activities or relative order of efficiency of the phenolic compounds from fermented *C. intermedia* (except hesperetin and hesperidin) to scavenge $O_2^{\bullet-}$ are not altered when compared on a molar basis (Table 2). The activity of hesperidin (IC_{50} = 1981.54) on a molar basis, is comparable to the poor activity of naringenin (IC_{50} = 1892.58), whereas hesperetin (IC_{50} = 2913.93) is extremely ineffective in scavenging $O_2^{\bullet-}$.

The relative decreasing order of efficiency of the test samples to scavenge $O_2^{\bullet-}$, as well as the magnitude of scavenging $O_2^{\bullet-}$, differs from their ability to scavenge $DPPH^{\bullet}$. There was no significant difference ($P \geq 0.05$) between the $O_2^{\bullet-}$ scavenging activities of the aqueous extract from fermented *C. intermedia* (IC_{50} = 238.21) and the methanol fraction prepared from the unfermented material (IC_{50} = 226.05), while the latter fraction showed a higher efficiency to scavenge $DPPH^{\bullet}$ than the aqueous extract (Table 2). No major difference occurred in the relative order of efficiency of the phenolic compounds

as scavengers of DPPH$^{\bullet}$ and $O_2^{\bullet-}$. The relative efficiency of mangiferin to scavenge $O_2^{\bullet-}$ was much lower than its H-donating ability towards DPPH$^{\bullet}$, to such an extent that it was even less effective than the aqueous extract of fermented *C. intermedia*.

In contrast to the H-donating ability, a decrease of *ca.* 64% was observed in the $O_2^{\bullet-}$ scavenging ability of luteolin when compared to quercetin on a molar basis (Table 2), which demonstrates the importance of the 3-OH group and its impact on $O_2^{\bullet-}$ scavenging. The absence of the 3-OH group from flavanones and flavones has a decreasing effect on $O_2^{\bullet-}$ scavenging capacity (Sichel *et al.*, 1991). According to Jovanovic *et al.* (1994), the highest reaction rates with $O_2^{\bullet-}$ were determined for the 3',4'-dihydroxyflavones (eg. quercetin), which have the lowest reduction potential of all investigated flavone radicals. Furthermore, the favourable electron-donating properties of quercetin is indicative of the efficient coupling between the B-ring radical with the 3-OH (or O^-) group in the C-ring through the 2,3-double bond (Jovanovic *et al.*, 1996). The 3',4'-dihydroxy configuration in the B-ring in combination with the 5-OH and 4-carbonyl groups in the A- and C-rings, respectively, are responsible for the efficiency of luteolin and eriodictyol to scavenge $O_2^{\bullet-}$. In the present study, the absence of the 2,3-double bond in the structure of eriodictyol did not affect its $O_2^{\bullet-}$ scavenging activity compared with luteolin ($P \geq 0.05$).

The importance of the 3',4'-dihydroxy configuration in the B-ring for effective radical scavenging is once again illustrated by the flavanone naringenin lacking this group, resulting in $O_2^{\bullet-}$ scavenging activity that was *ca.* 14 times weaker than that of the flavanone eriodictyol on a molar basis. The absence of the 3',4'-dihydroxy configuration, probably also account for both the incompetency of naringenin to scavenge DPPH$^{\bullet}$ and its extremely poor $O_2^{\bullet-}$ scavenging activity comparable to that of hesperidin on a molar basis. Jovanovic *et al.* (1994) who measured a high reduction potential for hesperidin phenoxyl radicals, showed that the lowest reaction rates with $O_2^{\bullet-}$ were observed for B-ring monohydroxy-substituted derivatives with substantially higher redox potentials.

Formononetin, flemichapparin, medicagol, 4-coumaric acid, as well as BHA and BHT were not effective scavengers of $O_2^{\bullet-}$, due to the absence of the structural features that is essential for effective radical scavenging. Nakamura *et al.* (1998) showed that 4-coumaric acid was ineffective towards enzymatically generated $O_2^{\bullet-}$ even at concentrations as high as 250 µM. Robak & Gryglewski (1988) showed that BHA

(1 - 300 µM) was inactive as a $O_2^{\bullet-}$ scavenger, while Zhou & Zheng (1991) also failed to indicate $O_2^{\bullet-}$ scavenging activity for BHA.

Inhibition of linoleic acid peroxidation

Aqueous extracts, crude phenolic fractions and phenolic compounds from *C. intermedia*, the reference compound, quercetin, and the synthetic antioxidants, BHA and BHT, inhibited linoleic acid peroxidation with varying degrees of efficiencies (Table 3). The overall relative order of inhibition of linoleic acid peroxidation by the test samples was as follows: BHT ≈ BHA ≈ luteolin ≈ isomangiferin ≈ eriodictyol ≈ mangiferin ≈ hesperetin > quercetin ≥ FM ≈ UM ≈ UD ≈ FD ≈ FA ≥ hesperidin ≈ 4-coumaric acid ≈ UA. Naringenin, formononetin, flemichapparin and medicagol were ineffective (Table 3). The inhibitory effect of FM, UM, FD and UD, and phenolic compounds from fermented *C. intermedia* on conjugated diene formation in a linoleic acid emulsion over time, is illustrated in Figs 3 and 4, respectively.

All aqueous extracts and crude phenolic fractions showed inhibition of linoleic acid peroxidation ranging between 58.5 and 70.3% ($P \geq 0.05$) (Table 3) when compared on an equal mass basis. Fermentation did not significantly affect the inhibitory effect of aqueous extracts and crude phenolic fractions of *C. intermedia* towards linoleic acid peroxidation ($P \geq 0.05$). In addition, selective extraction had no significant effect on the inhibition of linoleic acid peroxidation (Table 3). These phenomena occurred regardless of the variation in the total polyphenol content (Table 1). Black and green tea (*Camellia sinensis*) showed no significant differences in inhibiting low-density lipoprotein (LDL) oxidation (Hodgson *et al.*, 1999) regardless of the considerable differences in phenolic composition (Balentine *et al.*, 1997).

The activities of all extracts and fractions corresponded with that of hesperidin (63.3%) and 4-coumaric acid (60.7%). Of all extracts and fractions, only the unfermented aqueous extract of *C. intermedia* (UA, 58.5%) showed inhibition of linoleic acid peroxidation significantly different from that of quercetin (75.3%) ($P \leq 0.05$). Overall, the aqueous extracts and crude phenolic fractions showed weaker inhibition of linoleic acid peroxidation than phenolic compounds due to possible antagonistic effects by other tea components (eg. trace metals) (Frankel *et al.*, 1997).

Table 3 Inhibition of CD hydroperoxide formation in a linoleic acid emulsion by phenolic compounds from fermented *C. intermedia*, and aqueous extracts (FA, UA) and crude phenolic fractions from fermented and unfermented *C. intermedia* (FM, UM, FD and UD), compared with quercetin, BHA and BHT

Test material	**Inhibition (%) of CD hydroperoxide formation** [1,2]
BHT [3]	98.1 a
BHA	97.6 a
Quercetin	75.3 b
Luteolin [4]	97.4 a
Isomangiferin	94.9 a
Eriodictyol	94.1 a
Mangiferin	93.8 a
Hesperetin	91.5 a
Hesperidin	63.3 c
4-Coumaric acid	60.7 c
Naringenin	ineffective
Formononetin	ineffective
Flemichapparin	ineffective
Medicagol	ineffective
FM [5]	70.3 bc
FD	66.4 bc
UM	69.7 bc
UD	67.7 bc
FA [6]	66.2 bc
UA	58.5 c

[1] Inhibition (%) of CD hydroperoxide formation in the reaction medium calculated after 21 h incubation.

[2] Each value represents the means of three replicates (n = 3) with duplicate analysis. Means within the column followed by the same letter are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[3] Reference compounds: BHA, BHT and quercetin.

[4] Phenolic compounds present in fermented *C. intermedia*.

[5] Crude phenolic fractions from fermented and unfermented *C. intermedia*.

[6] Extraction of *ca.* 20 g tea leaves with 500 mL of freshly boiled deionised water for 5 min.

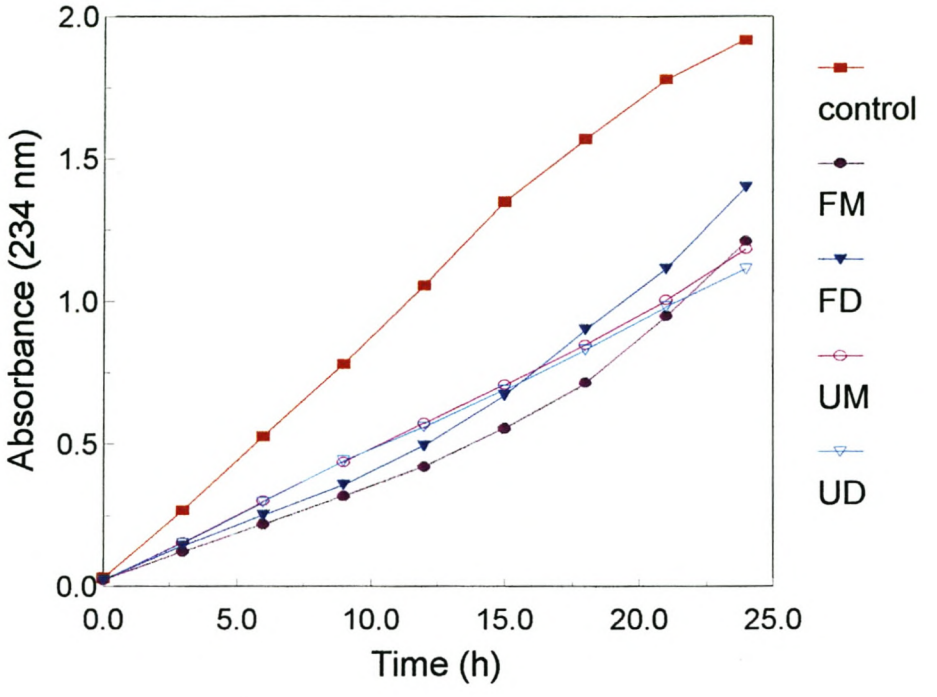

Figure 3 Efficiency of crude phenolic methanol (FM and UM) and diethyl ether (FD and UD) fractions from fermented and unfermented *C. intermedia* in comparison with a control, to inhibit linoleic acid peroxidation in a linoleic acid emulsion as monitored at 3 h-intervals for 21 h at 234 nm.

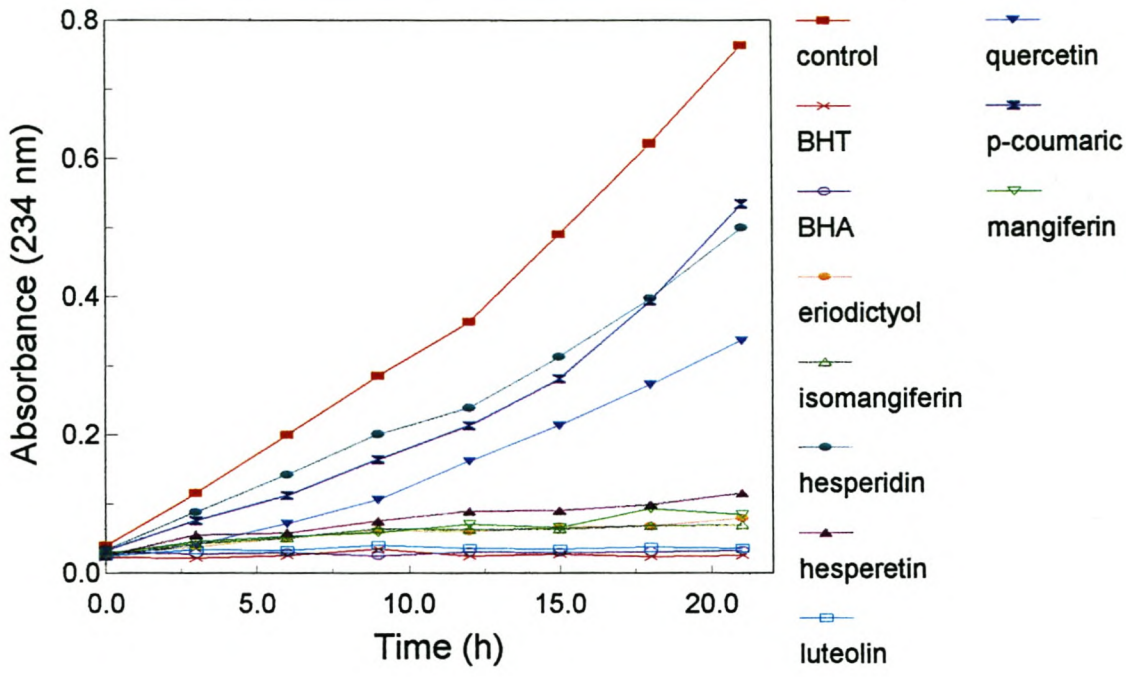

Figure 4 Efficiency of phenolic compounds from fermented *C. intermedia* in comparison with BHA, BHT and a control, to inhibit linoleic acid peroxidation (CD hydroperoxide formation) in a linoleic acid emulsion as monitored at 3 h-intervals for 21 h at 234 nm.

Luteolin, eriodictyol, mangiferin, isomangiferin, hesperetin, hesperidin and 4-coumaric acid inhibited linoleic acid peroxidation with varying efficiencies when compared on an equal mass (m v^{-1}) basis of 9.50 mg L^{-1} in the final reaction mixture (Table 3). Butylated hydroxyanisole (BHA), BHT, luteolin, eriodictyol, mangiferin, isomangiferin and hesperetin inhibited linoleic acid peroxidation with more than 90% ($P \geq 0.05$). The flavanone hesperidin and the hydroxy-cinnamic acid 4-coumaric acid with the weakest activity ($P \leq 0.05$), were still relatively effective at 63.3 and 60.7%, respectively. Individual polyphenols may inhibit lipid peroxidation by acting as chain-breaking antioxidants, thereby scavenging lipid alkoxyl and peroxyl radicals by means of H donation (Rice-Evans, 1995). It is expected that compounds showing excellent H-donating abilities (Table 2) would contribute to their inhibitory action towards linoleic acid peroxidation, which was indeed the case. Luteolin and eriodictyol, both very effective in scavenging DPPH$^{\bullet}$ and $O_2^{\bullet-}$, were also very effective in inhibiting linoleic acid peroxidation (> 90%) ($P \geq 0.05$). Mangiferin, with poor $O_2^{\bullet-}$ scavenging ability, but highly efficient scavenging of DPPH$^{\bullet}$, gave 93.8% inhibition of linoleic acid peroxidation. However, Sato *et al.* (1992) showed that mangiferin had no effect on terminating the radical chain reaction during the lipid peroxidation in microsomes, or in a linoleic acid hydroperoxide-induced peroxidation system. Mangiferin therefore does not have the ability to scavenge peroxy radicals involved in lipid peroxidation, but it possesses antioxidant activity probably due to its ability to scavenge free radicals involved in initiation of lipid peroxidation (Sato *et al.*, 1992). It was further suggested that mangiferin possibly exhibits its antioxidant activity by the catechol (6,7-dihydroxylated) moiety in its structure. The polyphenols naringenin, formononetin, medicagol and flemichapparin that were ineffective or poor scavengers of DPPH$^{\bullet}$ and $O_2^{\bullet-}$, were also ineffective towards inhibition of linoleic acid peroxidation. 4-Coumaric acid showed 60.7% inhibition of linoleic acid peroxidation, but was ineffective as scavenger of both DPPH$^{\bullet}$ and $O_2^{\bullet-}$.

For the remainder of the test samples, the ability to scavenge DPPH$^{\bullet}$ and/or $O_2^{\bullet-}$ (Table 2) did not correlate with the inhibitory action of linoleic acid peroxidation (Table 3). The reference compound quercetin and FM, respectively the strongest and poorest scavengers of $O_2^{\bullet-}$, inhibited CD hydroperoxide formation with 75.3 and 70.3%, respectively ($P \leq 0.05$). The very poor $O_2^{\bullet-}$ and moderate DPPH$^{\bullet}$ scavenging activities

of hesperetin are in contrast with its excellent inhibition of linoleic acid peroxidation (> 90%). This is in agreement with the data reported by Yuting *et al.* (1990), which showed no correlation between the antioxidative and radical scavenging activities of flavonoids. For instance morin and hispidulin showed very strong inhibition of lipid peroxidation regardless of very weak radical scavenging activity.

Discrepancies in efficiency between inhibition of linoleic acid peroxidation and scavenging of DPPH$^{\bullet}$ or $O_2^{\bullet-}$ as observed for the test samples indicate that other mechanisms, such as scavenging of OH$^{\bullet}$ or chelating of metal ions (Yuting *et al.*, 1990), may also contribute to the inhibition of linoleic acid peroxidation by flavonoids. Another essential consideration is the accessibility of the antioxidant to the radicals in question (Rice-Evans, 1999). In a lipid-containing system such as emulsions, antioxidants can partition between the hydrophobic lipid phase, the hydrophilic aqueous phase and the interfacial environment (Schwarz *et al.*, 1996). Antioxidant activity of compounds in a multi-phase system is affected by polarity, solubility, diffusion rates, stability, degree of dissociation, reactivity towards lipid radicals and tendency of their peroxyl and alkoxyl radicals to form secondary products (Huang *et al.*, 1996).

Lipophilic (non-polar) antioxidants show greater activity in polar lipid emulsions by being orientated in the oil-water interface, whereas the hydrophilic antioxidants remain solubilized in the aqueous phase (Porter *et al.*, 1989). The flavonoid aglycones are, for instance, more lipophilic (hydrophobic) with a higher affinity for the lipophilic phase than the respective hydrophilic flavonoid glycosides (Rice-Evans, 1995; Miyake *et al.*, 1997b). The marked difference in activity between the more hydrophilic hesperidin, showing *ca.* 30% weaker antioxidant activity compared with its more lipophilic aglycone, hesperetin, may not only be due to a concentration effect, but also to the partitioning of hesperidin into the aqueous phase, where it is less effective than the interfacially located hesperetin. Miyake *et al.* (1997a,b) furthermore illustrated that the antioxidant activity of the aglycones eriodictyol, hesperetin and naringenin was stronger than that of the respective flavonoid glycosides eriocitrin, hesperidin and naringin when compared on an equal molar concentration of 100 µM. In this particular study, lipid peroxidation was induced by 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) in an *in vitro* liposome system. A similar trend was observed when monitoring CD formation using methyl linoleate as an oxidizing substrate, as the sugar moiety in flavonol glycosides had a

marked decreasing effect on their antioxidant activity (Hopia & Heinonen, 1999). Yuting *et al.* (1990) illustrated that hesperidin did not inhibit lipid peroxidation of mouse liver homogenate, even with concentrations as high as 2000 μM. Furthermore, the high antioxidant activity of hesperetin towards inhibition of linoleic acid peroxidation could possibly be explained by increased lipophilicity resulting from methylation of the 4'-OH group, as was shown for the inhibitory effect of isoflavonoids towards microsomal lipid peroxidation (Jha *et al.*, 1985). Thus, the methoxy substitution in position 4' did not decrease the inhibitory action of hesperetin towards linoleic acid peroxidation, compared with eriodictyol.

Prior & Cao (1999) showed that flavonoids with multiple OH substitutions (eg. quercetin, luteolin and eriodictyol) are effective scavengers of peroxyl radicals. In a rabbit erythrocyte membrane model with autoxidation (Miyake *et al.*, 1997a) and the liposome system with AAPH-induced lipid peroxidation (Miyake *et al.*, 1997b), compounds containing adjacent hydroxy groups on the B-ring such as eriodictyol, showed high antioxidant activity. In this study, quercetin was less effective than luteolin, isomangiferin, mangiferin, eriodictyol and hesperetin towards inhibition of linoleic acid oxidation, probably due to the presence of the 3-OH group. Torel *et al.* (1986) indicated that the absence of the 3-OH group in the structure of luteolin is responsible for its stronger antioxidant activity against linoleic acid peroxidation in an aqueous medium, when compared with quercetin on an equal mass basis (10 μg mL^{-1}). Mangiferin is highly effective in inhibiting linoleic acid peroxidation, even with the presence of 2-β-*D*-glucopyranosyl, supposedly due to the multiple OH substitutions in the structure. The poor inhibitory action of formononetin in a microsomal lipid peroxidation system is attributed to the presence of the single OH-group at position 7 of its isoflavone structure (Jha *et al.*, 1985) and could also explain the poor activity in the present study.

The weaker activity of 4-coumaric acid towards linoleic acid peroxidation in comparison with the more effective phenolic compounds, is probably due to its monophenolic character making it less susceptible to antioxidant defense than the more efficient polyphenols (Chimi *et al.*, 1991). The highly lipophilic character of BHA and BHT (Vinson *et al.*, 1995) explains the highly effective antioxidant activity found for both these compounds in the present study.

Conclusions

The results discussed in this Chapter indicate that several honeybush tea polyphenols show potential as natural antioxidants, which warrant further investigation as a safer alternative to synthetic antioxidants, BHA and BHT. The efficiency of phenolic compounds from fermented *C. intermedia* to donate H to the stable free radical, $DPPH^{\bullet}$ and to scavenge $O_2^{\bullet-}$ is highly dependent on their structural arrangement. In both the $DPPH^{\bullet}$ and $O_2^{\bullet-}$ test systems, the reference compound quercetin was the most effective compound. The most effective honeybush tea polyphenol in both test systems was luteolin. Both quercetin and luteolin conform with the structural requirements for effective radical scavenging, i.e. the 3',4'-dihydroxy configuration in the B-ring combined with the 5-OH and 4-carbonyl groups in the A- and C-rings. Oxidation of the phenolic compounds during fermentation is probably the main cause for the decrease in the antiradical activity of FA, FM and FD, compared with their unfermented equivalents. This was illustrated, using HPLC analysis, by the decrease in the mangiferin/isomangiferin content of *C. intermedia* after fermentation. However, fermentation had little effect on the inhibition of linoleic acid peroxidation. The relationship between structure and activity of different compounds was not so profound in the linoleic acid assay, as most compounds showed the same percentage inhibition towards linoleic acid oxidation regardless of their structural differences.

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Chapter 5

Effect of particle size and extraction time on the superoxide scavenging and hydrogen-donating abilities of *Cyclopia maculata* (*ex* Du Toitskloof)

Abstract

The effects of particle size and extraction time on the efficiency of aqueous extracts prepared from the fermented material of *Cyclopia maculata* (*ex* Du Toitskloof) to scavenge 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) radicals, were investigated. Aqueous extracts prepared from tea material with a particle size $\leq$ 2 mm were more effective scavengers of $DPPH^{\bullet}$ and $O_2^{\bullet-}$ than aqueous extracts prepared from tea material with a particle size > 2 mm with a higher woody stem content. The lower activity of the latter extracts was attributed to the soluble solids containing less total polyphenols. Furthermore, the increased extraction of total polyphenols with increasing extraction times resulted in improved scavenging of $DPPH^{\bullet}$ and $O_2^{\bullet-}$.

Introduction

The traditional preparation method of honeybush tea from the indigenous fynbos plant *Cyclopia* involved prolonged brewing necessitated by the coarse processed ("fermented") plant material as a result of inadequate cutting equipment and the high percentage of woody stems. This preparation procedure was acceptable, especially since some consumers believed the unrefined product to have certain health-giving properties (Du Toit *et al.*, 1998). However, the modern consumer favours convenience

products that need minimum preparation time without sacrificing on the beneficial properties. With this concept in mind, Du Toit & Joubert (1998) introduced pre-wetting of the unfermented plant material prior to fermentation to improve steeping. Following the current interest from the industry in producing instant honeybush tea, the importance of retaining maximum antioxidant activity for the functional food market has to be considered.

It was only until recently that physical appearance of the final product became important due to consumer preferences (N. Coetzee, Coetzee & Coetzee Distributers, Cape Town, South Africa, personal communication, 2000). Sieving is, therefore, recommended to remove the coarser and mainly woody honeybush material in order to obtain a product with smaller particle sizes. Although a finer product is preferable in most cases, *ca.* 40% of honeybush tea is currently sold unrefined on the overseas market. Concept regulations for export markets makes provision for five different particle sizes, i.e. extra coarse cut (XCC), regular coarse cut (RCC), regular fine cut (RFC), super fine cut (SFC) and dust 1 (DT1). For instance, the RCC contains mostly particles with a diameter > 2 mm, whereas the RFC consists of mainly particles ≤ 2 mm (N. Coetzee, Coetzee & Coetzee Distributers, Cape Town, South Africa, personal communication, 2000). Aqueous extracts prepared from coarse material have relatively low soluble solid and total polyphenol contents as a consequence of the slow extraction rates of these substances from the coarser particles. The woody stems present in this material not only contribute to the low extraction values, but also to the low total polyphenol content of the soluble solids (Du Toit *et al.*, 1998).

It was concluded from data obtained in Chapter 3 of this dissertation that antioxidant activity of honeybush tea varies considerably with species, but is also negatively influenced by processing. The question that now arises and which is of major concern is: what is the impact of particle size (≤ 2 vs. > 2 mm) variation and the time of extraction on the antioxidant activity of the tea infusions? Therefore, to ensure maximum release of antioxidants from fermented material during brewing, the impact of extraction time (5, 15, 30, 45 and 60 min) and particle size (i.e. plant material ≤ 2 and > 2 mm) on antioxidant activity of aqueous extracts of fermented *C. maculata* (*ex* Du Toitskloof) was investigated. These extracts were compared for their scavenging abilities of the stable free radical, $DPPH^{\bullet}$ and $O_2^{\bullet-}$.

Materials and methods

Experimental procedures used in this study, except where noted otherwise, have already been described in Chapter 3 of this dissertation.

Harvesting and processing of plant material

Three different bundles of *C. maculata* (*ca.* 3 - 8 kg each), representing three replicates, were harvested in March 1997 from an experimental plantation in the Du Toitskloof area, which was established in 1995. The different bundles of honeybush tea were processed separately according to the standardised processing procedure described by Du Toit & Joubert (1998). After cutting the individual bundles into smaller pieces with a modified fodder cutter, *ca.* 2 kg of the cut leaves and stems from each bundle were moistened with deionised water followed by fermentation at 70°C for 60 h in a laboratory oven. The fermented material was dried at 40°C for 12 h in a drying tunnel (Decon Humidifier, Continental Fan Works cc., Cape Town, South Africa). The dried tea was sieved with an Endecotts test sieve (2 mm) (Endecotts (Filters) Ltd., London, England) to obtain a sieved fraction with a high leaf content (≤ 2 mm) and a coarser fraction (> 2 mm) containing more stems than the former fraction. Approximately 100 g of each fraction (≤ 2 and > 2 mm) were pulverized using a laboratory hammermill (Serial no 401, Scientific RSA).

Preparation of aqueous extracts

Aqueous extracts were prepared from test materials by pouring freshly boiled deionised water (500 mL) on *ca.* 20 g of tea leaves (pulverized (≤ 2 and > 2 mm); unpulverized (≤ 2 and > 2 mm)) followed by extraction in sealed vacuum flasks for 5, 15, 30, 45 and 60 min, respectively. The extracts were strained directly into 500 mL-volumetric flasks, rapidly cooled in an ice bath to room temperature, adjusted to volume with deionised water and filtered through Whatman No. 54 filter paper, before determination of soluble solid contents. Aliquots of the filtrates were stored in specimen containers at -20°C until needed for analysis.

Statistical analysis

Analysis of variance (ANOVA) (SAS Release version 6.12) was used to determine whether extraction time and particle size significantly affected the parameters tested. Pairwise Student's t-tests were used to compare means when an effect was significant ($P \leq 0.05$). Regression analysis was performed on data obtained for extraction time to test for significant trends in this regard.

Results and discussion

Soluble solid, total polyphenol and flavonoid contents of aqueous extracts

The soluble solid contents of aqueous extracts from fermented *C. maculata* (*ex* Du Toitskloof) were found to increase with increasing extraction times ($P \leq 0.05$) for both fractions (≤ 2 and > 2 mm) (Table 1). Water pretreatment of the honeybush tea material, a critical step of the standardised processing method, resulted in partial diffusion of the soluble solids to the surface of the tea leaves (Du Toit & Joubert, 1998). Similar to that from rooibos tea (Joubert & Hansmann, 1990), these solutes were rapidly extracted from the leaf surface in contact with water, leading to an initial rapid increase in the soluble solid concentration as found with the 5 min extraction (Table 1). Depletion of solutes on the leaf surface, as well as slow diffusion of some solutes (eg. larger molecules and less soluble substances) from the leaf matrix, resulted in the decreasing rate of extraction. However, prolonged exposure of the tea material to water will aid in the diffusion of such compounds from the particles.

The total polyphenol and flavonoid contents of aqueous extracts were found to rapidly increase ($P \leq 0.05$) during the first 30 min of extraction. For extraction periods exceeding 30 min, the total polyphenol contents of the aqueous extracts slightly increased whilst the flavonoid contents remained constant ($P \geq 0.05$) (Table 1). The total polyphenols expressed on a soluble solid basis (Table 2) followed the same trend as the total polyphenols of the aqueous extracts (Table 1). Diffusion rates of polymeric substances, eg. polyphenols are slower than those of smaller molecules (Joubert, 1990) and would therefore benefit from the increased extraction time.

Table 1 The effect of extraction time and particle size on the soluble solid, total polyphenol and flavonoid contents of aqueous extracts of fermented *C. maculata* (*ex* Du Toitskloof) material

Extraction time (min)	Soluble solids [1,2]		Total polyphenols [1,3]		Flavonoids [1,3]	
	≤ 2 mm	> 2 mm	≤ 2 mm	> 2 mm	≤ 2 mm	> 2 mm
5	0.351 a	0.225 a	0.032 a	0.017 a	0.009 a	0.004 a
15	0.453 b	0.305 b	0.044 b	0.026 b	0.018 b	0.007 b
30	0.500 c	0.356 c	0.053 c	0.032 c	0.024 c	0.013 c
45	0.502 d	0.384 d	0.055 cd	0.037 cd	0.026 c	0.015 c
60	0.521 e	0.402 e	0.057 d	0.040 d	0.028 c	0.017 c

[1] Each value represents the means of three replicates (n = 3). For particles ≤ 2 and > 2 mm, respectively, the means of the soluble solids, total polyphenols and flavonoids, followed by the same letter is not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[2] Concentration expressed as g soluble solids 100 mL^{-1} aqueous extract.

[3] Concentration expressed in terms of g GAE 100 mL^{-1} aqueous extract.

Table 2 The effect of extraction time and particle size on the total polyphenol content and antiradical activities, i.e. H-donating (EC_{50} value) and $O_2^{\bullet-}$ scavenging (IC_{50} value), of aqueous soluble solids of fermented *C. maculata* (*ex* Du Toitskloof) material

Extraction time (min)	Total polyphenols [1,2]		EC_{50} [1,3]		IC_{50} [1,4]	
	≤ 2 mm	> 2 mm	≤ 2 mm	> 2 mm	≤ 2 mm	> 2 mm
5	9.117 a	7.556 a	35.0 a	45.2 a	309.3 a	319.2 a
15	9.713 b	8.525 b	32.8 b	39.7 b	270.0 b	307.5 b
30	10.600 c	8.989 c	30.2 c	34.4 c	246.2 c	279.4 c
45	10.956 cd	9.635 cd	27.6 cd	32.4 cd	231.9 c	265.7 c
60	10.940 d	9.950 d	26.3 d	28.8 d	216.9 d	249.6 d

[1] Each value represents the means of three replicates (n = 3) (with duplicate analysis only for total polyphenols). For particles ≤ 2 and > 2 mm, respectively, the means of the total polyphenol and flavonoid contents of the soluble solids, followed by the same letter, are not significantly different ($P \geq 0.05$) as determined by pairwise Student's t-tests.

[2] Concentration expressed in terms of g GAE 100 g^{-1} soluble solids.

[3] Expressed as µg soluble solids 50 μL^{-1} aqueous tea extract in the reaction mixture necessary to decrease initial [DPPH$^{\bullet}$] by 50%.

[4] Expressed as µg soluble solids mL^{-1} reaction mixture required to inhibit NBT reduction by $O_2^{\bullet-}$ with 50%.

The soluble solid, total polyphenol and flavonoid contents of the sieved fraction (≤ 2 mm) was higher ($P \leq 0.05$) than that of the coarse fraction (> 2 mm) (Table 1). Although slower diffusion of solutes from larger particles (> 2 mm) would attribute to lower extraction values, and *vice versa*, the possibility might exist of compositional changes between the smaller (≤ 2 mm) and larger (> 2 mm) particles.

For aqueous extracts prepared from the pulverized material of both fractions (≤ 2 and > 2 mm), the total polyphenol content of the soluble solids remained unchanged either with increasing extraction times or different particle sizes (Fig. 1). This indicates that the total polyphenols originally present in the plant material were maximally and easily extracted from the very small particles of the powdered material into the water. The rate of diffusion was thus not a limiting factor, as was the case with the coarse material (> 2 mm). Furthermore, this indicates that the expected compositional differences between the fraction containing smaller particles (≤ 2 mm) and the fraction containing more woody material (> 2 mm), are not adequately discriminated by the total polyphenol assay.

DPPH$^{\bullet}$ and superoxide anion radical ($O_2^{\bullet-}$) scavenging abilities

The DPPH$^{\bullet}$ and $O_2^{\bullet-}$ scavenging abilities of *C. maculata* (*ex* Du Toitskloof) increased with increasing extraction time for both fractions (≤ 2 and > 2 mm) ($P \leq 0.05$) as indicated by decreasing EC_{50} and IC_{50} values, respectively (Table 2). This could be attributed to increasing total polyphenol content ($P \leq 0.05$) of the aqueous extracts with increasing extraction times (Table 2). Extraction of polyphenols with a higher affinity to scavenge DPPH$^{\bullet}$ and $O_2^{\bullet-}$, would also contribute to this effect. The decrease in the EC_{50}/total polyphenol or IC_{50}/total polyphenol ratios (Fig. 2) substantiates this conclusion.

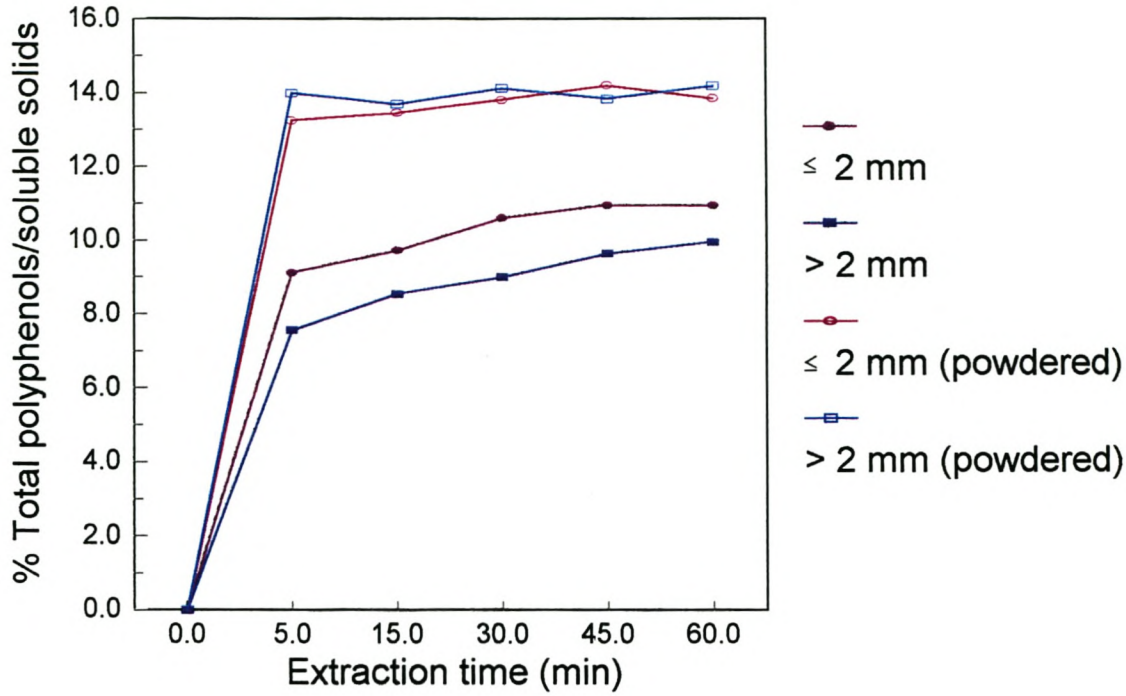

Figure 1 The effect of increasing extraction times and particle sizes (pulverized (≤ 2 and > 2 mm); unpulverized (≤ 2 and > 2 mm)) on the total polyphenol (TP) content, expressed as a percentage of the soluble solids of aqueous extracts prepared from fermented *C. maculata* (*ex* Du Toitskloof).

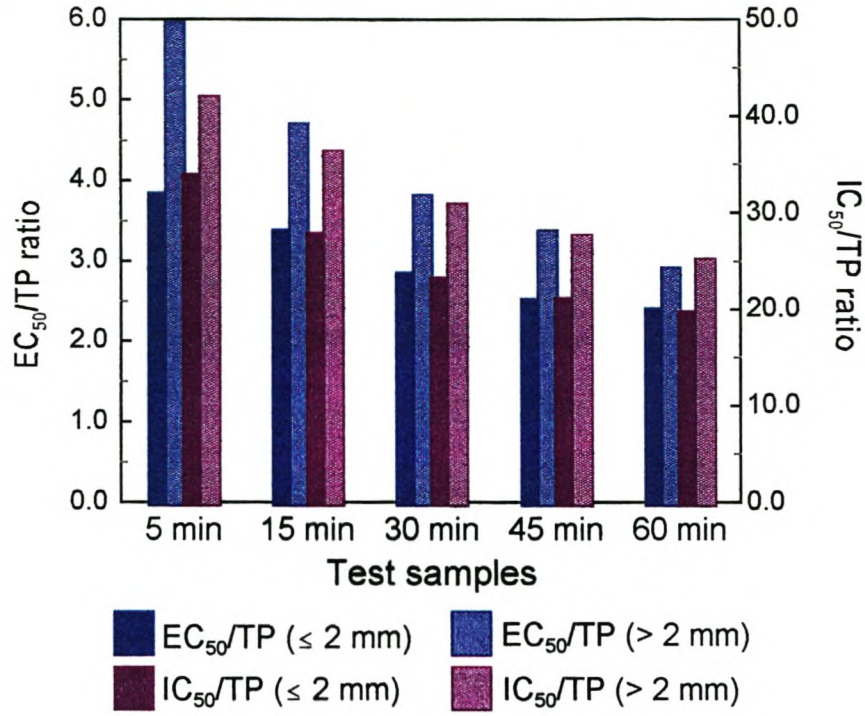

Figure 2 The hydrogen-donating (EC_{50} values) and superoxide scavenging (IC_{50} values) activities of aqueous extracts of *C. maculata* (*ex* Du Toitskloof) expressed as a ratio of the total polyphenol (TP) contents of the soluble solids. Extracts were prepared from plant material (≤ 2 and > 2 mm) using varying extraction times (5, 15, 30, 45 and 60 min).

The fraction containing particles with a smaller diameter (≤ 2 mm) showed both higher hydrogen-donating (H-donating) (lower EC_{50} values) and $O_2^{\bullet-}$ scavenging abilities ($P \leq 0.05$) than extracts prepared with coarser material (Table 2). Once again, the difference in total polyphenol content (Table 2) and the relative efficiency of the phenolic compounds (Fig. 2) would explain this. This could be attributed to the fact that phenolic metabolites and flavonoids are more easily diffused into water from particles with a smaller diameter (≤ 2 mm) than from coarser particles (> 2 mm).

Prolonged brewing of the woody material increased the antioxidant activity to such an extent that it correlated well with the activities of the sieved material. The 30-min extraction of the woody material had a very similar H-donating ability to the 5 min-extraction of the sieved fraction (≤ 2 mm). The 15 min-extraction prepared from the woody material (> 2 mm) correlated with the 5-min extraction of the sieved material (≤ 2 mm) in terms of the $O_2^{\bullet-}$ scavenging activity.

Conclusion

The overall consumer favours shorter brewing times when preparing tea as it is less time-consuming. This may be accomplished by using tea material with a smaller particle size. This study showed that honeybush tea infusions prepared using shorter brewing times still had sufficient antiradical potential. It was also shown that the antioxidant or antiradical benefits of honeybush tea may be enhanced by increasing brewing times. Furthermore, infusions prepared from material > 2 mm containing more woody stems exhibit antiradical activity, but is less effective in scavenging $DPPH^{\bullet}$ and $O_2^{\bullet-}$ than infusions prepared using smaller particles (≤ 2 mm). The data obtained in this study is of importance especially since the modern consumer is becoming more aware of the health benefits of foods and teas in particular. A shorter brewing time without losing antiradical potential would be seen as a great health and convenience advantage.

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Chapter 6

General discussion and conclusions

Honeybush tea is a traditional herbal infusion that was mainly prepared for home consumption using an indigenous fynbos plant (*Cyclopia* spp.) (Du Toit *et al.*, 1998). A 50% increase in production and consumption over the past year (N. Coetzee, Coetzee & Coetzee Distributers, Cape Town, South Africa, personal communication, 2000) gives ample evidence of the demand for this product. The association of honeybush tea with certain health claims (Du Toit *et al.*, 1998), research into the development of a standardised processing method (Du Toit & Joubert, 1998) and the fact that it is pure, natural and caffeine-free, are all factors contributing to the increasing popularity of this herbal beverage. Furthermore, its potential as a functional beverage was realized following the characterization and isolation of phenolic compounds from fermented *C. intermedia*, of which some are associated with certain pharmacological properties (Ferreira *et al.*, 1998). A follow-up study by Kamara (1999) revealed the presence of additional phenolic compounds in the fermented tea. The possible *in vivo* toxicity of synthetic chemicals (Aruoma *et al.*, 1990; Kim *et al.*, 1994; Yen & Duh, 1994) and an increasing consumer preference for natural products (Bondet *et al.*, 1997) provided a substantial basis for the *in vitro* quantification of the antioxidant and antiradical scavenging activities of honeybush tea. The variation in the phenolic constituents of different species (De Nysschen *et al.*, 1996), as well as the declining total polyphenol content of honeybush tea extracts during fermentation due to chemical oxidation (Du Toit & Joubert, 1998), suggested a potential impact on the antioxidant status of honeybush tea.

In the present study aqueous extracts prepared from the unfermented and fermented stems and leaves of *C. intermedia*, *C. genistoides*, *C. maculata*, *C. sessiliflora* and *C. subternata* were used to assess the influence of processing ("fermentation") on *in vitro* antioxidant and antiradical potential of these species. It was evident from the data that the antiradical efficiencies of the aqueous extracts depended to a large degree on the total polyphenol content. This was substantiated by the

aqueous extracts from unfermented *C. sessiliflora*, of which the soluble solids contained the highest total polyphenol contents, showing the best scavenging of both 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) radicals. The strong antiradical efficiency explains the strong inhibition of the latter extract towards linoleic acid peroxidation. On the contrary, fermented *C. subternata* for which the soluble solids contained the lowest total polyphenol contents, showed the poorest hydrogen-donating (H-donating) ability, moderate scavenging of $O_2^{\bullet-}$ and very good inhibition towards linoleic acid peroxidation. The soluble solids of fermented *C. maculata* and *C. intermedia*, also showing low total polyphenol and flavonoid contents, consequently exhibited the weakest $O_2^{\bullet-}$ scavenging, weak H-donating ability to $DPPH^{\bullet}$ and the poorest inhibition of linoleic acid peroxidation. This variation in efficiencies emphasizes the need for the use of different test systems to assess the antioxidant and antiradical potential of honeybush tea infusions. The contradictions observed between the antiradical efficiencies of the soluble solids of different species in different test systems are attributed to qualitative differences in their phenolic composition. This indicates that specific compounds in the extracts, rather than the total polyphenol contents, are important for effective antiradical activities.

Soluble solids of extracts prepared from unfermented material contained more total polyphenols than those from fermented material. Subsequently the unfermented material had higher antiradical potential than the fermented equivalent. Oxidation of the phenolic compounds during fermentation was probably the main cause for the decrease in the antiradical activity of the fermented fractions in comparison with the unfermented equivalents. HPLC analysis indeed showed a decrease in the (iso-)mangiferin content of a methanol fraction from *C. intermedia* with fermentation. Both mangiferin and isomangiferin exhibited strong scavenging of $DPPH^{\bullet}$ and $O_2^{\bullet-}$. Fermentation had little effect on the efficiency of aqueous extracts and crude phenolic fractions in inhibiting linoleic acid peroxidation, except for *C. intermedia* where the unfermented aqueous extract showed weaker activity than the fermented equivalent. *Cyclopia genistoides* showed the strongest inhibition of linoleic acid peroxidation, while unfermented *C. intermedia* showed the weakest inhibitory activity against linoleic acid peroxidation. It should be considered that this *in vitro* testing of linoleic acid peroxidation may differ from the actual oxidation process in the human body. It is, therefore, essential to

measure the antioxidant activity of the honeybush tea test material using *ex vivo* or *in vivo* experiments, eg. inhibiting peroxidation of membranes such as erythrocytes, liposomes or microsomes.

Preliminary *in vitro* experiments conducted with the present study have shown the inhibitory effect of fermented and unfermented *C. intermedia* towards the xanthine oxidase (XOD) enzyme. This could possibly benefit the human body in that less harmful reactive intermediates, including $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2), are produced in human tissue during the normal oxidation process of xanthine to uric acid (Gutteridge & Halliwell, 1994). It is recommended that the inhibitory effect of honeybush tea on this enzyme is further investigated.

Another aim of the present study was to determine the effect of fractionation on the antioxidant and antiradical potential of fermented and unfermented *C. intermedia*. Fractions were selectively prepared from this material in an effort to improve the antioxidant and antiradical activities of aqueous extracts. The total polyphenol content of the aqueous soluble solids of fermented and unfermented *C. intermedia*, respectively, decreased and increased by *ca.* 28% during selective extraction with methanol. However, further fractionation of the methanol fractions did not affect the total polyphenol content for both unfermented and fermented *C. intermedia*. The H-donating ability of the aqueous extracts of fermented *C. intermedia* decreased with selective extraction, while an improvement in the H-donating ability was observed for the unfermented material. For both fermented and unfermented *C. intermedia*, the $O_2^{\bullet-}$ scavenging activities of the aqueous extracts decreased with selective methanol extraction, but again increased during fractionation of the methanol fractions with diethyl ether. The possibility therefore exists that the antioxidant and antiradical efficiencies of honeybush tea aqueous extracts might be improved or optimized by fractionation for further application in the functional food and cosmetic industries. However, regardless of the variation in the total polyphenol content, selective extraction had no significant effect on the inhibitory effects of aqueous extracts and crude fractions towards linoleic acid peroxidation.

The importance of the structural arrangement of the phenolic compounds isolated from fermented *C. intermedia* to efficiently scavenge free radicals, i.e. $DPPH^{\bullet}$ and $O_2^{\bullet-}$, was illustrated in the present study. The antiradical efficiencies observed for these

compounds were in agreement with numerous other studies using similar or different radical scavenging systems. Quercetin, an ubiquitous plant flavonoid and food antioxidant, was used as reference compound, since previous studies illustrated its potency as natural antioxidant (Formica & Regelson, 1995). In this particular study, the honeybush tea polyphenol, luteolin, was found to be slightly less effective in scavenging $DPPH^{\bullet}$ and $O_2^{\bullet-}$ than quercetin. Both quercetin and luteolin conform with all the structural requirements for effective radical scavenging as previously suggested by Bors *et al.* (1990), i.e. the 3',4'-dihydroxy configuration in the B-ring combined with the 5-OH and 4-carbonyl groups in the A- and C-rings. Luteolin and several honeybush tea polyphenols thus show potential as possible natural alternatives to synthetic antioxidants in order to retard lipid peroxidation in foods and cosmetics. The isoflavone formononetin and the coumestans, flemichapparin and medicagol, did not scavenge $DPPH^{\bullet}$ and $O_2^{\bullet-}$ and were also ineffective towards inhibition of linoleic acid peroxidation. Although the latter polyphenols were inefficient as antioxidants and radical scavengers in the present study, isoflavones and coumestans possess phyto-oestrogenic activity (Verdeal & Ryan, 1979).

The relationship between structure and activity of different compounds was not so profound in the linoleic acid assay, as most compounds showed the same degree of inhibition towards linoleic acid oxidation regardless of their structural differences. A possible explanation for this phenomenon was the partitioning of the compounds between the hydrophobic lipid phase, the hydrophilic aqueous phase and the interfacial environment. In such a case, the antioxidant efficiency of the compounds are dependant on several factors, i.e. polarity, solubility, diffusion rates, stability and degree of dissociation (Huang *et al.*, 1996). Furthermore, the involvement of mechanisms other than H-donating ability or scavenging of $O_2^{\bullet-}$ is suggested for inhibition of linoleic acid peroxidation, eg. scavenging of $OH^{\bullet}$ or chelation of metal ions (Yuting *et al.*, 1990).

The final aim of the present study was to determine whether particle size and extraction time affected the antioxidant and antiradical activities of honeybush tea. For this purpose, aqueous extracts were prepared from the material of *C. maculata* (*ex* Du Toitskloof). From the data obtained, it was concluded that aqueous extracts prepared from tea material with a particle size $\leq$ 2 mm were more effective scavengers of $DPPH^{\bullet}$ and $O_2^{\bullet-}$ than aqueous extracts prepared from tea material > 2 mm. The weaker activity

of the latter extracts was attributed to the soluble solids containing less total polyphenols resulting from the higher percentage of woody stems. The 5 min-extraction prepared from the coarser material (> 2 mm) exhibited the weakest antiradical activity. However, prolonged brewing of this material to at least 15 min will exhibit antiradical activity corresponding to the 5 min-extraction prepared from tea material ≤ 2 mm. The antiradical activities of tea infusions may be enhanced by increasing extraction times. This is a result of the increased extraction of total polyphenols from the tea material with prolonged brewing. The modern consumer, who prefers shorter preparation times, could therefore still enjoy the full health benefit when using tea material with smaller particles (≤ 2 mm).

Tea fermentation, which mainly involves oxidation of the phenolic compounds, is essential for the development of the characteristic sensory properties of honeybush tea, i.e. dark brown colour and the "honey-like" flavour. This oxidation process was, however, found to be detrimental for the antiradical activities of all the *Cyclopia* species tested. For instance, unfermented honeybush aqueous extracts showed more effective scavenging of $DPPH^{\bullet}$ and $O_2^{\bullet-}$ than the fermented equivalents. For maximum antiradical potential, fermentation of honeybush tea is, therefore, not advisable. From a consumer's perspective, fermentation of honeybush tea material is recommended for improved taste and flavour, thereby possibly compromising on the health properties. Flavourants may be used to improve the grassy flavour of infusions prepared from unfermented honeybush material. Another possibility is the use of honeybush tea in its unfermented form as a natural antioxidant in lipid-containing foodstuffs and cosmetics, in an effort to prolong their shelf-life. In the nutraceutical market, the poor flavour of unfermented honeybush tea may be masked by incorporating in food products, such as herbal drinks.

In an effort to validate the antioxidant and antiradical activities of honeybush tea (*Cyclopia*), as illustrated by the preliminary *in vitro* tests used in this study, further *in vitro* and *ex vivo* testing is essential for extensive characterization of the radical scavenging potential of the individual phenolic compounds. Finally, testing of honeybush tea using *in vivo* models is crucial, as well as testing of the bioavailability of the phenolic compounds following consumption of tea infusions by human subjects in clinical trials. Another important consideration in the evaluation of the antioxidant and

antiradical potential of honeybush tea is the characterisation and quantification of the phenolic profile of other species, apart from that of fermented *C. intermedia*. Fermented *C. intermedia*, the only species for which the phenolic profile has been characterised (Ferreira *et al.*, 1998) due to its commercial importance, was unfortunately the species showing the weakest antioxidant and antiradical activities compared to some of the other species tested in this study. Nevertheless, the successful marketing of honeybush tea in the food and cosmetic industries, and the already competitive functional food (nutraceutical) market, is entirely dependant on sound scientific evidence of its efficiency as a natural antioxidant in foods and cosmetics, and its benefits for human consumption. Is honeybush tea perhaps the elixir of life we have all been waiting for?

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