

A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas

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Abstract

Antimutagenic activity of aqueous extracts of the South African herbal teas, *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush) was compared with that of *Camellia sinensis* (black, oolong and green) teas in the *Salmonella* mutagenicity assay using aflatoxin B₁ (AFB₁) and 2-acetylaminofluorene (2-AAF) as mutagens. The present study presents the first investigation on antimutagenic properties of *C. subternata*, *C. genistoides* and *C. sessiliflora*. The herbal teas demonstrated protection against both mutagens in the presence of metabolic activation, with the exception of “unfermented” (green/unoxidised) *C. genistoides* against 2-AAF, which either protected or enhanced mutagenesis depending on the concentration. Antimutagenic activity of “fermented” (oxidised) rooibos was significantly ($P < 0.05$) less than that of *Camellia sinensis* teas against AFB₁, while for 2-AAF it was less ($P < 0.05$) than that of black tea and similar ($P > 0.05$) to that of oolong and green teas. Antimutagenic activity of unfermented *C. intermedia* and *C. subternata* exhibited a similar protection as fermented rooibos against AFB₁. Against 2-AAF, fermented rooibos exhibited similar protective properties than unfermented *C. intermedia* and *C. sessiliflora*. Unfermented rooibos was less effective than the *C. sinensis* teas and fermented rooibos, but had similar ($P > 0.05$) antimutagenicity to that of fermented *C. sessiliflora* against AFB₁ and fermented *C. subternata* against 2-AAF. Fermented *C. intermedia* and *C. genistoides* exhibited the lowest protective effect against 2-AAF, while fermented *C. intermedia* exhibited the lowest protection when utilising AFB₁ as mutagen. Aspalathin and mangiferin, major polyphenols in rooibos and *Cyclopia* spp., respectively, exhibited weak to moderate protective effects when compared to the major green tea catechin, (–)epigallocatechin gallate (EGCG). Antimutagenic activity of selected herbal tea phenolic compounds indicated that they contribute towards (i) observed antimutagenic activity of the aqueous extracts against both mutagens and (ii) enhancement of the mutagenicity of 2-AAF by unfermented *C. genistoides*. Antimutagenic activity of the South African herbal teas was mutagen-specific, affected by fermentation and plant material, presumably due to changes and variation in phenolic composition.

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1. Introduction

A modern lifestyle is associated with frequent and unavoidable exposure to carcinogenic risk factors, e.g. cigarette smoke, chronic infections and inflammation, nutrition and dietary factors, and exposure to environmental chemicals [1], which necessitate neutralisation and/or protection against their deleterious effects [2]. In this regard identification of chemopreventive compounds in commonly consumed natural dietary sources, such as fruits, vegetables and tea, is of particular importance in that it provides means of everyday cancer prevention [3].

A number of studies during the last decade have linked tea (*Camellia sinensis*) consumption, especially green tea, to a reduced risk for cancer in humans [4–6], leading to increased popularity and credibility of tea as a health drink with chemopreventive properties. Conversely, consumer concern regarding the detrimental effects of caffeine on health has increased consumption of decaffeinated teas and the development in sophisticated and health conscious markets has led to herbal teas being consumed as alternatives to other beverages [7]. The demand for the indigenous South African herbal tea, rooibos (*Aspalathus linearis* (Burm. Fil) R. Dahlgr. spp. *linearis*), by the international market has increased to such an extent that the tonnage currently exported exceeds domestic use [8]. Honeybush tea produced from different *Cyclopia* spp. is another herbal tea indigenous to South Africa that made the transition from limited localised use to commercial cultivation to meet the demand of the export market. Popularity and consumption of this beverage is currently growing and more than 80% of production is exported [9]. Despite the absence of caffeine in both these herbal teas [10,11] and several health promoting properties associated with their consumption [12–14], information regarding their antimutagenic and anticancer properties is limited and becoming increasingly important.

The antimutagenic activity of aqueous extracts prepared from “fermented” (oxidised) and “unfermented” (green/unoxidised) rooibos against metabolically activated 2-AAF has been demonstrated [15]. Marnewick et al. [16,17] reported that aqueous extracts from fermented and unfermented rooibos and honeybush (*C. intermedia*) possess *in vitro* and *ex vivo* antimutagenic activity against the promutagens (require metabolic activation) 2-AAF and AFB₁. These extracts, however, offered no or only weak protection against the direct-acting mutagens, methylmethane sulfonate (MMS), cumolhydroperoxide (CHP) and hydrogen peroxide (H₂O₂) [16]. Aqueous extracts, prepared from both fermented and unfermented

rooibos and *C. intermedia*, modulate some of the phase II drug-metabolising enzymes, reduce oxidative stress and enhance the antioxidant capacity of rat liver after chronic exposure [18]. In a recent study the inhibitory effect on skin cancer development by fermented and unfermented rooibos and honeybush (*C. intermedia*) extracts was demonstrated in a two-stage mouse skin carcinogenesis assay [19].

In the present study the protective effect of aqueous extracts of fermented and unfermented honeybush and rooibos was compared with that of *C. sinensis* teas, i.e. black (fermented), oolong (semi-fermented) and green (unfermented), in the *Salmonella* mutagenicity assay, using AFB₁ and 2-AAF as mutagens. Selection of mutagens was based on the frequent use of 2-AAF as model carcinogen, representative of cooked food mutagens and nitroaromatic compounds [20], and AFB₁, as a food-borne mycotoxin known to be a potent liver carcinogen in human populations [21–23]. Both these mutagens require metabolic activation, but the pathways leading to the reactive mutagenic intermediates differ [20,24]. The antimutagenic properties of selected phenolic compounds, present in the respective herbal teas, were also assessed. These compounds included the dihydrochalcone aspalathin, the principal monomeric flavonoid in unfermented rooibos [25–27] and a major compound in fermented rooibos [28,29], and mangiferin, a xanthone found in high levels in commercially important *Cyclopia* spp. [30]. The antimutagenic activity of the most abundant catechin, (–)epigallocatechin gallate (EGCG), and the major antimutagenic principle in green tea [31], which is also a phenolic constituent of unfermented *C. subternata* [32], was included for comparative purposes.

2. Materials and methods

2.1. Chemicals

Luteolin (>90%), eriodictyol (HPLC grade), chrysoeriol (HPLC grade), eriocitrin (HPLC grade) and narirutin (HPLC grade) were purchased from Extrasynthèse (Genay, France). (–)–Epigallocatechin gallate (EGCG) from green tea (>95%), mangiferin from *Mangifera indica* leaves, hesperidin (97%), hesperetin (≥95%), AFB₁, β-nicotinamide adenine dinucleotide phosphate (β-NADP), bovine serum albumin (BSA) and dimethyl sulfoxide (Fluka DMSO for UV-spectroscopy) (≥99.8%) were obtained from Sigma–Aldrich Chemicals Co. (Cape Town, South Africa). Aspalathin (≥95% as determined by HPLC and LC–MS) was isolated from unfermented rooibos tea at the PROMEC Unit of the Medical Research Council (MRC, Bellville, South Africa) (Snijman et al., unpublished data). The tester strains, *Salmonella typhimurium* TA100 and TA98 and Aroclor 1254, were obtained from Dr. B.N. Ames

(Berkeley University, California, USA and from Monsanto (St. Louis, USA), respectively).

2.2. Preparation of samples

2.2.1. Plant material

Six samples (2–5 kg), comprising more than one plant, of each *Cyclopia* spp. were randomly collected at different geographical locations in the Western Cape Province of South Africa. *Cyclopia intermedia* was harvested (March 2000) in the Haarlem area, while *C. subternata* was harvested during February and October 1999 from 3- and 4-year-old plantations in the Outeniqua area and in February 1999 from a 2-year-old plantation in the Du Toitskloof area. *C. genistoides* (West Coast type) was harvested (March 2001) from a 5-year-old plantation in the Overberg region and *C. sessiliflora* was obtained from Helderfontein, the ARC experimental farm, Stellenbosch (February 2001). The harvested *Cyclopia* plant material was processed according to the standardised processing procedure of Du Toit and Joubert [33] into fermented and unfermented counterparts. The leaves and stems of each of the species were cut into small pieces (≤ 4 mm) using a modified fodder cutter. The shredded plant material was moistened with deionised water (water treated with a Modulab Water Purification System Separations, Cape Town) and fermented at 70 °C for 60 h. The fermented material was then 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 in a drying tunnel (40 °C for 12 h) directly after shredding. The dried material, fermented and unfermented (<10% moisture content; wet basis), was sieved with an Endecott test sieve (2 mm) (London, England) and the fraction containing mostly leaves and small pieces of stems (≤ 2 mm) was pulverised in a Retsch rotary mill (1 mm sieve).

Unfermented *A. linearis* was prepared from six samples (ca. 100 g) collected from individual plants in the rooibos production area between Citrusdal and Nieuwoudtville, from 1-, 2- and 5-year-old plantations between 1997 and 1999. Unfermented material was prepared following the procedure described for *Cyclopia* spp. with the exception that the shoots were dried intact to prevent cell damage and consequent oxidation. Fermented *A. linearis* samples were randomly collected at the processing facility of Rooibos Ltd., Clanwilliam during 1999. Six samples of each of the *C. sinensis* teas (green, black and oolong) were purchased from commercial retailers in South Africa. The plant material was stored in airtight containers in a cool, dark place until use.

2.3. Preparation of aqueous extracts

Aqueous extracts were prepared by steeping 100 g of milled tea (1-mm sieve) in 1000 mL freshly boiled deionised water for 5 min. Extracts were coarse-filtered with a Buchner filter, using a 125- μ m synthetic mesh cloth (Polymer PES D25/35 supplied by Swiss Silk Bolting Cloth Mfg. Co. Ltd, Zürich,

Switzerland), followed by filtration with Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England) to remove fine particles. The filtrates were freeze-dried in an Atlas pilot-scale freeze-drier (Denmark model, Copenhagen, Denmark, 40 °C shelf temperature) after being frozen at –20 °C in plastic trays (170 mm \times 115 mm \times 30 mm). The dried filtrates were subsequently removed from the trays and stored in screw cap glass vials, desiccated in the dark at room temperature.

2.4. Antimutagenicity assay

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

A liver homogenate fraction was prepared from Aroclor 1254-induced male Fischer rats, according to the method of Maron and Ames [34]. The induction procedure involved dilution of Aroclor 1254 in sunflower oil to a concentration of 200 mg mL⁻¹ and administration of a single intraperitoneal injection of 500 mg kg⁻¹ to each Fischer rat (ca. 200 g) 5 days before sacrifice [35]. The rat liver homogenate was centrifuged (Sorvall Superspeed RC2-B centrifuge, Separations Scientific, South Africa) at 9000 \times g for 10 min and aliquots of the supernatant (S9 fraction) were stored in glass vials at –80 °C until used. The protein concentration was determined according to the method described by Kaushal and Barnes [36] with bovine serum albumin as standard. The cytochrome P450 content was assessed using the method of Omura and Sato [37]. The S9 mixture contained the S9 fraction (2 mg protein mL⁻¹/0.7 nM cytochrome P450 mg⁻¹ protein), MgCl₂–KCl salts (8 mM MgCl₂, 33 mM KCl), 5 mM glucose-6-phosphate, 4 mM NADP and 0.1 M phosphate buffer (pH 7.4), made up to a final volume of 50 mL with sterile distilled water.

2.4.2. Antimutagenicity of aqueous tea extracts and selected phenolic compounds

Antimutagenic activity of the freeze-dried aqueous extracts from *C. sinensis* and herbal teas against AFB₁ and 2-AAF in tester strains TA100 and TA98, respectively, was determined as percentage inhibition according to the standard plate-incorporation assay [34]. Antimutagenicity was assessed by adding 0.1 mL of the mutagen (20 ng of AFB₁ or 5 μ g of 2-AAF per plate), 0.1 mL of the different concentrations of the tea extracts [0.1%, 0.5%, 1%, 2% and 5% (w/v)], 0.5 mL S9 mixture and 0.1 mL of an overnight bacterial culture to 2 mL of molten top agar, containing 0.05 mM biotin–histidine. The mixture was dispersed onto minimal glucose agar plates and incubated at 37 °C for 48 h. Stock solutions of AFB₁ and 2-AAF were freshly prepared using DMSO as solvent. The aqueous extracts were dissolved in sterile water, diluted to the required concentration and filtered with a 0.45- μ m filter, followed by a 0.22- μ m filter (sterile Nylon Cameo filters, Separations, Cape Town, South Africa). Antimutagenic activity of the selected phenolic compounds (dissolved in DMSO) was determined at a concentration level of 0.3 mM per plate and included

aspalathin, luteolin, chrysoeriol, eriodictyol, hesperidin, hesperetin, narirutin, eriocitrin, mangiferin and EGCG.

Extracts and compounds that enhanced the mutagenicity of 2-AAF were tested for mutagenic activity in the absence of the mutagen, with and without metabolic activation (S9). Control treatments (negative control) included TA100 or TA98 in the presence of the S9 mixture to determine the spontaneous revertants, while positive control plates contained the S9 mixture, TA100 or TA98 and AFB₁ or 2-AAF, respectively. The number of spontaneous revertants was subtracted before calculating the percentage inhibition.

2.4.3. Statistical analysis

Statistical analyses was performed with SAS version 9.1 and included tests for normality among the groups with the Kolmogorov Smirnov Test and testing for homogeneity of group variances with Levene's Test. A one-way analysis of variance (ANOVA) was performed for testing significant group differences followed by a post hoc Tukey's Studentised range test, to determine which groups differed significantly. Where sample sizes were unequal, the Tukey–Cramér adjustments were made automatically. Statistical significance was considered at 5% ($P < 0.05$).

3. Results

3.1. Antimutagenicity of aqueous tea extracts

3.1.1. AFB₁ as a mutagen

All the *C. sinensis* and herbal teas exhibited protection against AFB₁-induced mutagenesis (Table 1) at the concentration levels tested. A significant ($P < 0.05$) dose–response effect was evident for *C. sinensis* teas at the lower concentration levels, with black and oolong teas demonstrating maximum inhibition at both 1 and 2 mg aqueous extract per plate. Antimutagenic activity of *C. sinensis* and herbal teas was subsequently compared at a concentration level of 0.5 mg aqueous extract per plate, where black tea demonstrated significantly ($P < 0.05$) higher antimutagenic activity than the green and herbal teas. Fermented rooibos exhibited similar protective properties as green and oolong tea. At a concentration of 0.1 mg aqueous extract per plate, the antimutagenic activity of fermented rooibos was significantly ($P < 0.05$) lower than that of the *C. sinensis*

Table 1

Antimutagenic activity of aqueous extracts prepared from fermented and unfermented *Cyclopia* spp. and rooibos, and black, oolong and green teas against AFB₁-induced mutagenesis using *Salmonella typhimurium* TA100

Tea/herbal tea mg extract per plate	% Inhibition			
	0.1 mg	0.5 mg	1 mg	2 mg
<i>C. sinensis</i>				
Black tea (fermented)	38.37 ± 10.83 ^a a ^{#b}	95.93 ± 6.64 a* (87.67–102.51) ^c	99.61 ± 5.71 a**	98.81 ± 5.36 a ^{***}
Oolong tea (semi-fermented)	31.70 ± 11.00 a [#]	88.65 ± 5.87 ab* (83.27–92.98)	97.23 ± 5.43 ab**	98.79 ± 11.22 a ^{**}
Green tea (unfermented)	33.4 ± 13.28 a [§]	81.82 ± 13.63 bc* (67.10–95.97)	92.11 ± 8.20 ab**	97.72 ± 6.73 a [#]
<i>A. linearis</i>				
Fermented	14.5 ± 11.97 b [§]	82.61 ± 8.15 bc* (71.77–89.65)	94.12 ± 5.55 ab**	98.12 ± 7.34 a [#]
Unfermented	nd ^d	63.74 ± 13.48 e* (52.16–85.34)	83.90 ± 13.99 c**	93.59 ± 7.98 ab [#]
<i>Cyclopia</i> spp.				
<i>C. intermedia</i>				
Fermented	n.d.	25.83 ± 12.43 i* (14.57–39.26)	50.18 ± 12.85 f ^{**}	79.21 ± 8.14 def [#]
Unfermented	n.d.	77.28 ± 7.32 cd* (70.26–85.76)	93.15 ± 6.43 ab**	97.28 ± 4.81 a [#]
<i>C. subternata</i>				
Fermented	n.d.	39.53 ± 12.56 gh* (25.96–57.33)	60.77 ± 12.29 e**	78.68 ± 12.48 def [#]
Unfermented	n.d.	73.66 ± 10.97 cd* (61.13–86.25)	90.48 ± 6.66 bc**	96.54 ± 4.75 a [#]
<i>C. genistoides</i>				
Fermented	n.d.	47.05 ± 13.54 fg* (29.50–61.04)	71.12 ± 7.57 d**	87.87 ± 7.02 bc [#]
Unfermented	n.d.	49.79 ± 9.8 f [§] (35.39–60.57)	67.08 ± 13.4 de**	83.24 ± 9.96 cde [#]
<i>C. sessiliflora</i>				
Fermented	n.d.	42.23 ± 15.14 fgh* (28.62–61.04)	65.78 ± 7.15 de**	85.61 ± 8.56 cd [#]
Unfermented	n.d.	71.79 ± 11.28 de* (63.19–79.15)	90.92 ± 8.02 bc**	98.58 ± 5.06 a [#]

Means in the same column followed by different letters are significantly different ($P < 0.05$).

^a Values represent the mean ± S.D. of percentage inhibition of six samples ($n = 6$) with five assay replications.

^b Means in the same row marked with different symbol uppercase (*, **, #, §) indicate a dose–response effect with significant differences ($P < 0.05$) between the concentration levels for the same tea.

^c Values in parenthesis represent the minimum and maximum inhibition demonstrated by the six individual samples of each respective tea.

^d Not determined.

^e The mean frequency of spontaneous reversion for the different assays, ranged between 104–134 with the mean AFB₁ (20 µg per plate) induced mutagenesis ranging between 333–429.

teas, which exhibited similar protective activities. Comparison of the herbal teas at 0.5 mg aqueous extract per plate showed that fermented rooibos had a similar protective effect to unfermented *C. intermedia* and *C. subternata*, but offered a higher ($P < 0.05$) protection than the other fermented and unfermented *Cyclopi*a spp. Unfermented rooibos demonstrated a weaker ($P < 0.05$) protection than unfermented *C. intermedia* and *C. subternata*, which was all similar to unfermented *C. sessiliflora*, but higher ($P < 0.05$) than unfermented *C. genistoides* and the different fermented *Cyclopi*a spp.

Regarding fermentation, both black tea and fermented rooibos demonstrated significantly ($P < 0.05$) higher antimutagenic activity than green and unfermented rooibos, respectively, at a concentration level of 0.5 mg extract per plate. Fermentation significantly ($P < 0.05$) reduced the antimutagenic activity of *Cyclopi*a spp., except for *C. genistoides* where no significant difference ($P > 0.05$) was noted. Fermented *C. intermedia* exhibited the lowest ($P < 0.05$) antimutagenic activity at levels of

0.5 and 1 mg aqueous extract per plate when compared with the other *Cyclopi*a spp.

3.1.2. 2-AAF as a mutagen

All the *C. sinensis* teas and herbal teas, with the exception of unfermented *C. genistoides*, exhibited protection against 2-AAF-induced mutagenesis (Table 2), although the percentage inhibition was lower than for AFB₁-induced mutagenesis, at equal extract concentrations. A significant ($P < 0.05$) dose–response effect was evident for the teas, except for black and oolong teas at 2 and 5 mg aqueous extract per plate, where a maximum level of inhibition was observed. Black tea exhibited a significantly ($P < 0.05$) higher antimutagenic activity than the herbal teas at all concentrations tested. At 0.5 mg aqueous extract per plate, antimutagenic activity of black tea was significantly ($P < 0.05$) higher than that of oolong tea, which exhibited significantly ($P < 0.05$) higher activity than green tea. At this concentration fermented rooibos, oolong and green tea exhibited similar ($P > 0.05$)

Table 2

Antimutagenic activity of aqueous extracts prepared from fermented and unfermented *Cyclopi*a spp. and rooibos, and black, oolong and green teas against 2-AAF-induced mutagenesis in *S. typhimurium* TA98

Tea/herbal tea mg extract per plate	% Inhibition			
	0.5 mg	1 mg	2 mg	5 mg
<i>C. sinensis</i>				
Black tea (fermented)	82.19 ± 5.15 ^a a ^{#b}	102.39 ± 1.51 a [*]	102.29 ± 1.11 a ^{***} (101.24–103.02) ^c	102.23 ± 1.73 ab ^{***}
Oolong tea (semi-fermented)	59.39 ± 8.25 b ^{**}	n.d.	101.94 ± 2.19 ab [*] (99.63–103.16)	103.32 ± 2.64 a [*]
Green tea (unfermented)	48.55 ± 11.93 c [*]	77.35 ± 6.89 b ^{**}	96.01 ± 4.33 abc [#] (91.89–100.19)	
<i>A. linearis</i>				
Fermented	53.28 ± 11.69 bc [*]	75.33 ± 6.03 bc ^{**}	93.29 ± 6.40 cde [#] (84.63–100.10)	100.23 ± 3.43 bc ^{\$}
Unfermented	nd ^d	35.75 ± 20.27 f ^{**}	72.81 ± 19.79 h ^{**} (52.68–98.68)	99.28 ± 2.59 cd [#]
<i>Cyclopi</i> a spp.				
<i>C. intermedia</i>				
Fermented	n.d.	n.d.	53.95 ± 8.57 i [*] (45.61–63.01)	91.13 ± 5.03 f ^{**}
Unfermented	n.d.	72.79 ± 8.73 bcd [*]	93.64 ± 5.19 bcd ^{**} (85.94–98.94)	
<i>C. subternata</i>				
Fermented	n.d.	n.d.	76.00 ± 6.99 fgh [*] (69.06–97.27)	96.15 ± 2.67 e ^{**}
Unfermented	n.d.	54.85 ± 15.12 e [*]	83.20 ± 8.99 f ^{**} (69.07–94.49)	
<i>C. genistoides</i>				
Fermented	n.d.	n.d.	50.24 ± 10.21 i [*] (40.85–56.99)	85.86 ± 6.37 g ^{**}
Unfermented	20.18 ± 11.00 d [*]	(+) ^e 14.65 ± 20.66 g ^{**}	(+) 43.53 ± 20.86 j [#] (–58.86 to –9.46)	
<i>C. sessiliflora</i>				
Fermented	n.d.	n.d.	82.75 ± 4.74 fg [*] (79.20–89.03)	98.68 ± 2.49 cde ^{**}
Unfermented	n.d.	65.39 ± 7.77 d [*]	93.30 ± 5.79 cde ^{**} (85.35–102.25)	

Means in the same column followed by different letters are significantly different ($P < 0.05$).

^a Values represent the mean ± S.D. of percentage inhibition of six samples ($n = 6$) with five assay replications.

^b Means in the same row marked with different symbol uppercase (*, **, #, \$) indicate a dose–response effect with significant differences ($P < 0.05$) between the concentration levels for the same tea.

^c Values in parenthesis represent the minimum and maximum inhibition demonstrated by the six individual samples of each respective tea.

^d Not determined.

^e An enhancing effect of 2-AAF-induced mutagenesis.

^f The mean frequency of spontaneous reversion, for the different assays, ranged between 25–44 with the mean 2-AAF (5 µg per plate) induced mutagenesis ranging between 232–490.

Table 3

Mutagenicity testing with and without metabolic activation of unfermented *C. genistoides* and phenolic compounds in the *Salmonella* mutagenicity assay

Tea extract/compound	+S9	–S9
Unfermented <i>C. genistoides</i> (1 mg per plate)	40 ± 4 ^a	34 ± 2
Unfermented <i>C. genistoides</i> (2 mg per plate)	41 ± 5	40 ± 3
Eriodictyol	40 ± 3 ^b	34 ± 2
Hesperidin	34 ± 4	28 ± 5
Narirutin	30 ± 4	29 ± 3
Control (+) ^c	423 ± 41	
Control (–) ^c	32 ± 6	

^a Values represent the mean number of revertants for five assay replications with (+S9) and without (–S9) the metabolic activation in TA98 of unfermented *C. genistoides* at two concentration levels.

^b Values represent the mean number of revertants for three assay replications with (+S9) and without (–S9) in TA98 of the compounds (0.3 mM per plate) that demonstrated an enhancing effect of the mutagenicity of 2-AAF.

^c The mean frequency of spontaneous reversion [control (–)] and the mean revertant count for 2-AAF (5 µg per plate) induced mutagenesis [control (+)].

protection. Fermented rooibos, unfermented *C. intermedia* and *C. sessiliflora* displayed similar ($P > 0.05$) protection to green tea at 1 and 2 mg aqueous extract per plate, but a significantly ($P < 0.05$) higher protective effect than unfermented rooibos and *C. subternata*. Unfermented *C. genistoides* exhibited relatively weak (20.18%) antimutagenic activity at 0.5 mg extract per plate, while it enhanced the mutagenesis of 2-AAF at 1 and 2 mg extract per plate. However, it did not show a mutagenic response by itself, in the absence or presence of metabolic activation when using tester strain TA98 (Table 3).

Fermentation resulted in a significant ($P < 0.05$) reduction in the antimutagenic activity of *C. intermedia*, and *C. sessiliflora* but not *C. subternata*, when compared at a concentration of 2 mg aqueous extract per plate. In contrast, fermentation significantly ($P < 0.05$) enhanced the protective effect of *C. genistoides*. Fermented *C. genistoides* and *C. intermedia* exhibited similar ($P > 0.05$), but the lowest protective effects of the different *Cyclopia* spp.

3.1.3. Variation in antimutagenic activity as a result of variation in plant material

Significant ($P < 0.05$) variations in activity against AFB₁- and 2-AAF-induced mutagenesis were observed between samples of the same tea and herbal tea species. The minimum and maximum values of inhibition by the six individual samples of tea at concentrations of 0.5 mg (against AFB₁) and 2 mg (against 2-AAF) aqueous

extract per plate are indicated in Tables 1 and 2 (in parentheses), respectively.

The highest variation in antimutagenic activity against AFB₁ was evident for unfermented rooibos (52.16–85.34%) and fermented *C. subternata* (25.96–57.33%), *C. genistoides* (29.50–61.04%) and *C. sessiliflora* (28.62–61.04%). Antimutagenicity of unfermented rooibos (52.68–98.68% inhibition) and the enhancing effect of unfermented *C. genistoides* [(+) 9.46% to (+) 58.86% enhancement] also varied considerably against 2-AAF.

3.2. Antimutagenic activity of selected phenolic compounds

Antimutagenic activity of the selected phenolic compounds was compared at 0.3 mM per plate against metabolically activated AFB₁ and 2-AAF (Table 4).

EGCG, hesperetin, eriodictyol and chrysoeriol demonstrated similar ($P > 0.05$) antimutagenic activity (between 85.99% and 95.40% inhibition) against AFB₁, which was significantly ($P < 0.05$) higher than that of hesperidin, eriocitrin and mangiferin. Luteolin was less ($P < 0.05$) effective than EGCG, but had antimutagenic activity similar ($P > 0.05$) to that of hesperetin, eriodictyol and chrysoeriol. Narirutin (40.82%) and aspalathin (30.42%) demonstrated the weakest protection against AFB₁.

The order and potency as well as the effects for the respective compounds were different against 2-AAF-induced mutagenesis. Luteolin, followed by EGCG, exhibited significantly ($P < 0.05$) higher protection against 2-AAF-induced mutagenesis when compared to the other flavonoids. Aspalathin, eriocitrin and hesperetin demonstrated similar ($P < 0.05$), but relatively weak (less than 20% inhibition) protection, while narirutin, hesperidin and eriodictyol enhanced the mutagenicity. These compounds were, however, not mutagenic by themselves, when tested in the absence or presence of metabolic activation (Table 3).

4. Discussion

Green tea (*C. sinensis*) has well recognised antimutagenic properties against several mutagens [31,38–42] and was therefore included in this study to serve as a benchmark antimutagen. This investigation presents the first comparative study on *in vitro* antimutagenic activity of commercially important *Cyclopia* spp. and *A. linearis* relative to *C. sinensis* teas, in order to obtain information regarding the antimutagenic potency of rooibos and honeybush. Antimutagenicity of *C. subternata*, *C. genis-*

Table 4

Antimutagenic activity and structures of selected phenolic compounds (0.3 mM per plate) of *Cyclopia* spp., rooibos and *Camellia sinensis* teas against AFB₁ and 2-AAF in *S. typhimurium* TA100 and TA98, respectively

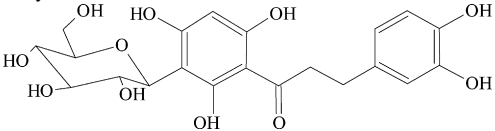
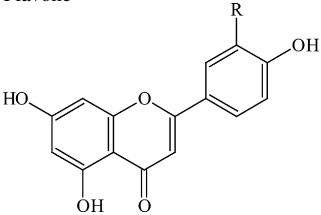
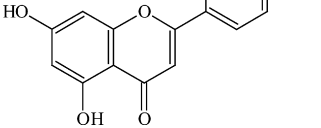
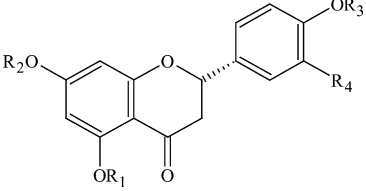
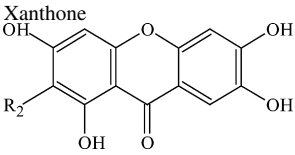
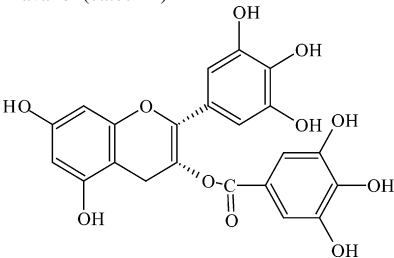
Compound type	Compound	Substitution	Source/tea	Antimutagenicity AFB ₁ ^a	Antimutagenicity 2-AAF ^a
Dihydrochalcone		Aspalathin	Unfermented and fermented rooibos [14,25,28]	30.43 ± 6.39 h	11 ± 4.05 e
Flavone		Luteolin R = OH	Unfermented and fermented rooibos [28,53], fermented <i>C. intermedia</i> [58], unfermented <i>C. subternata</i> [32]	82.49 ± 1.01 bcd	97.19 ± 3.16 a
		Chrysoeriol R = Ome	Unfermented and fermented rooibos [53,28]	85.99 ± 4.99 abcd	40.16 ± 5.48 c
Flavanone		Eriodictyol R ₁ = R ₂ = R ₃ = H, R ₄ = OH	Fermented <i>C. intermedia</i> [58]	88.39 ± 3.98 abc	(+) ^b 57.03 ± 5.43 h
		Hesperidin R ₁ = H, R ₂ = rutinosyl, R ₃ = Me, R ₄ = OH	Fermented and unfermented <i>C. intermedia</i> [30,58], unfermented <i>C. subternata</i> [32], unfermented <i>C. genistoides</i> and <i>C. sessiliflora</i> [30]	69.44 ± 3.66 e	(+) 34.86 ± 2.64 g
		Hesperetin R ₁ = R ₂ = H, R ₃ = Me, R ₄ = OH	Fermented <i>C. intermedia</i> [58]	91.55 ± 4.6 ab	6.29 ± 7.96 e
		Narirutin R ₁ = R ₃ = R ₄ = H, R ₂ = rutinosyl	Unfermented <i>C. subternata</i> [32]	40.82 ± 4.56 g	(+) 20.08 ± 9.03 f
		Eriocitrin R ₁ = R ₃ = H, R ₂ = rutinosyl, R ₄ = OH	Unfermented <i>C. subternata</i> [32]	68.21 ± 5.42 ef	10.04 ± 2.43 e

Table 4 (Continued)

Compound type	Compound	Substitution	Source/tea	Antimutagenicity AFB ₁ ^a	Antimutagenicity 2-AAF ^a
Xanthone 	Mangiferin	R ₂ = 2-β-D-glucopyranosyl	Fermented and unfermented <i>C. intermedia</i> [30,32,58], unfermented <i>C. genistoides</i> and <i>C. sessiliflora</i> [30]	66.35 ± 5.36 ef	26.99 ± 7.75 d
Flavanol (catechin) 	EGCG		Unfermented <i>Camellia sinensis</i> [54], unfermented <i>C. subternata</i> [32]	95.40 ± 2.70 a	66.51 ± 8.80 b

Means in the same column followed by different letters are significantly ($P < 0.05$) different.

^a Values represent the mean ± S.D. of percentage inhibition of five assay replications.

^b An enhancing effect of 2-AAF-induced mutagenesis.

^c The frequency of spontaneous reversion was 134 ± 6 (negative control) and 425 ± 11 (positive control) for determining antimutagenic activity against AFB₁ (20 μg per plate) and 29 ± 10 (negative control) and 366 ± 34 (positive control) for determining antimutagenic activity against 2-AAF (5 μg per plate).

toides and *C. sessiliflora* against AFB₁ and 2-AAF is also reported for the first time. A previous study on the antimutagenic activity of *A. linearis* and *C. intermedia* [16] included only one plant material sample. In the present study six samples of each tea were included to obtain natural variability.

The aqueous extracts of the *C. sinensis* and herbal teas demonstrated a varying degree of protection against the mutagenicity of the two procarcinogens, AFB₁ and 2-AAF, whilst under certain conditions, one of the *Cyclopia* spp. enhanced the mutagenic response of 2-AAF. The type of mutagen as well as the dose of the tea used, influenced antimutagenic activity. A higher protection was generally observed against AFB₁ than against 2-AAF-induced mutagenesis, which is in agreement with a study by Marnewick et al. [16], which reported that the protection demonstrated by *C. intermedia* and rooibos did not only depend on the type of tea and/or total polyphenol content, but also on the specific mutagen used. This was attributed to various pathways of metabolic activation of the carcinogens AFB₁ and 2-AAF and the possible differences in the effects by *C. intermedia* and rooibos in terms of their protection against the mutagens [16]. The metabolism of AFB₁ results in a single electrophilic AFB₁-8,9-epoxide [24], while the electrophilic products, derived during the two-step activation of 2-AAF, include *N*-hydroxy-2-AAF and the acetylated *N*-acetoxy-2-AAF [43].

Antimutagenic activity of green and black teas (*C. sinensis*) has been attributed to the actions of polyphenolic compounds, mainly catechins and their oxidised products, i.e. the theaflavins and thearubigins [44,45]. Dissimilar antimutagenic activity of the *C. sinensis* and herbal teas, compared in the present study, may thus be related to the differences in their polyphenolic profiles. Black tea (fermented *C. sinensis*) is rich in thearubigins and theaflavins, while green tea (unfermented *C. sinensis*) contains a considerable amount of monomeric polyphenols [40], which mainly belong to three subclasses: the flavan-3-ols, the flavonols and the flavones [46], with flavan-3-ols (catechins) being the predominant form. The major monomeric polyphenols in rooibos comprise the dihydrochalcones, aspalathin and nothofagin [30] and the flavones, orientin and *iso*-orientin [29], whereas in *Cyclopia* spp. they encompass the xanthones, mangiferin and isomangiferin and the flavanone, hesperidin [30]. Aspalathin and mangiferin exhibited weak to moderate protective effects when compared to the major green tea catechin, (–)epigallocatechin gallate (EGCG).

The other phenolic compounds from rooibos and *Cyclopia* spp., investigated in the present study, demon-

strated various levels of protection against the mutagenicity of AFB₁. Furthermore, a significantly lower inhibition was noticed against 2-AAF, with the exception of three flavanones that enhanced mutagenicity. Eriodictyol, hesperidin and narirutin, present in *Cyclopia* spp. [47–49] enhanced the mutagenicity of 2-AAF, suggesting that these phenolic compounds contribute towards the enhancement of 2-AAF mutagenesis by aqueous extracts of unfermented *C. genistoides*. However, the presence of the flavanones in the other *Cyclopia* spp. [47–49] did not result in enhancement of the mutagenicity of 2-AAF, implicating that different levels and/or combinations of phenolic compounds and/or other constituents may be involved in the inhibition/enhancement of mutagenicity. Variation in the levels of protection of phenolic compounds against different mutagens has been reported previously [50,51]. Luteolin, the only compound demonstrating potent antimutagenic activity (more than 80% inhibition) against both mutagens in the present study, is known to be a potent antimutagen against several mutagens [50,52,53]. Mangiferin may play a role in protection of the herbal teas against AFB₁, but is likely to contribute to a far lesser extent to antimutagenic activity against 2-AAF. In rooibos, aspalathin does not appear to be the principal compound responsible for antimutagenic activity, since it exhibited relatively low levels of protection against both mutagens. In this regard, luteolin and, to a certain extent, chrysoeriol, exhibited far higher antimutagenic activity, but is present at substantially lower levels in rooibos [29,54]. The contribution of relatively moderate antimutagens such as aspalathin, as opposed to the contribution of a potent antimutagen such as luteolin, towards the antimutagenic activity of rooibos remains to be elucidated. This is also relevant for *Cyclopia* spp., which contains mangiferin at relatively high levels, compared to the potent antimutagen, luteolin which is present in small quantities [49]. An important consideration is that the antimutagenic potency of compounds at the same molar concentration level (0.3 mM in the present study) should not be interpreted as a reflection of their possible contribution to the antimutagenic activity of tea, since actual levels in the teas and herbal teas differ and synergistic effects between the compounds were not taken into account.

Fermentation of the herbal and *C. sinensis* teas is known to have a profound effect on their qualitative and quantitative phenolic composition [28,55]. It also affected their antimutagenic potency as demonstrated in this study, again implicating the role of phenolic compounds. Black and green teas have been reported to have the same degree of antimutagenic activity, suggesting that green tea flavanols are similarly active when

compared with black tea polyphenols, theaflavins and thearubigins [56]. Conversely, black tea has also been found to be a more effective antimutagen against 2-AAF than green tea [57]. In the present study black tea exhibited a higher antimutagenic activity than green tea against both 2-AAF and AFB₁. Fermented rooibos also exhibited higher antimutagenic activity than unfermented rooibos against both mutagens, which was in contrast to the results of a study by Standley et al. [58], demonstrating that fermentation significantly ($P < 0.05$) reduced antimutagenic potency of rooibos against 2-AAF. This was associated with a reduction in the total polyphenolic content with fermentation [58], shown to result in substantially lower aspalathin and nothofagin contents of rooibos [26]. This discrepancy of the effect of fermentation on antimutagenic potency of rooibos may be attributed to sample size and sampling. In the present study samples consisted of 100 g of plant material selected from individual plants. The samples used by Standley et al. [58] consisted of 1 kg of plant material, collected from the respective processing stages at a commercial rooibos processing plant (Rooibos Ltd., Clainwilliam, South Africa), where plant material from more than one producer could have been blended together after shredding. Although the average antimutagenic activity of fermented rooibos was higher than that of the unfermented rooibos in the present study, some of the unfermented samples displayed higher antimutagenic activity. An important consideration would be that extracts prepared from fermented and unfermented *C. sinensis* and *A. linearis* were not prepared from the same plant material and therefore the differences in antimutagenic activity observed in the present study may not only be due to changes occurring with fermentation, but also due to sample selection.

In contrast, the same plant material sample was used in preparation of fermented and unfermented *Cyclopia* teas. The decreased antimutagenic activity of *Cyclopia* spp. with fermentation, except for *C. genistoides*, may be related to the reduction of the total polyphenol content [55], specifically that of mangiferin and hesperidin [48,49]. Fermentation of *C. genistoides* resulted in a change in its activity from enhancing the mutagenicity of 2-AAF to protection at the higher concentration levels (1 and 2 mg aqueous extract per plate). This effect may be attributed to the formation of antimutagenic compounds and/or a reduction in the enhancing constituents during fermentation. The enhancing effect of unfermented *C. genistoides* against 2-AAF-induced mutagenesis is concentration-dependent, since the aqueous extracts exhibited relatively weak antimutagenic activity at the lowest concentration, whilst enhancing

mutagenesis at higher concentrations. It is not known at present whether increased concentrations of the phenolic compounds will result in a typical dose–response effect or if these compounds may have a protective and/or enhancing effect against metabolically activated mutagens, depending on their concentration level. No significant difference was noticed between fermented and unfermented *C. genistoides* when using AFB₁ as mutagen, once again suggesting mutagen specificity. Of interest was that a similar response was noticed with the flavanones present in *Cyclopia* spp. when considering the enhancing mutagenic effect of 2-AAF and the protection against AFB₁-induced mutagenesis. The mechanism involved in the enhancement of the mutagenicity of 2-AAF by extracts of *C. genistoides* and some of the flavanones is not known at present. As these extracts and compounds are not mutagenic *per se*, the observed enhancement may be due to possible interaction of herbal tea constituents with the metabolic enzymes involved in the activation of the mutagen, possibly resulting in an increased concentration of the putative mutagenic metabolites. Studies are in progress to elucidate the mechanisms involved in antimutagenic activity as well as enhanced mutagenicity demonstrated by the herbal teas and phenolic compounds.

Significant ($P < 0.05$) differences existed in the antimutagenic activity of the different samples of plant material within the same *Cyclopia* spp., which may be attributed to quantitative variation in chemical composition. The samples used in the present study were collected from plants propagated from seeds and plants growing in the wild, thus resulting in large genetic variation. Other factors that could contribute to quantitative variation are age of the plantation, growth and leaf-to-stem ratio. The polyphenolic content, specifically that of mangiferin and hesperidin, of two types of *C. genistoides* harvested every 5 weeks over a 15-week period, varied [30]. Different levels of both aspalathin and nothofagin have been reported in the stems and leaves of rooibos, as well as in plant material collected in different areas [27]. The present study implies that the antimutagenic activities of the herbal teas may be affected by quantitative variation in chemical composition caused by factors already mentioned. As specific causative factors were not evaluated, future investigations are recommended to determine the influences of above-mentioned parameters on the antimutagenic potency of the herbal teas.

Antimutagenic activity of rooibos and *Cyclopia* spp. was comparable to that of extracts prepared from *C. sinensis*, suggesting that consumption of these two herbal teas may have beneficial health effects. Differences in the polyphenolic profiles, the protective effect

of phenolic compounds present in the herbal teas and the effect of fermentation on the activity suggest that these compounds play an important role in the antimutagenic activity of the herbal teas. Natural plant variation of rooibos and *Cyclopia* spp. markedly affects antimutagenic activity against AFB₁ and 2-AAF, emphasising the importance of sample selection when studying the biological activities of the herbal teas.

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