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The potential role of polyphenols in the modulation of skin cell viability by *Aspalathus linearis* and *Cyclopia* spp. herbal tea extracts *in vitro*

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Keywords
antioxidant properties; chemoprevention; herbal tea; polyphenols; skin cell viability

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Abstract

Objectives The relationship between polyphenol constituents, antioxidant properties of aqueous and methanol extracts of green tea (*Camellia sinensis*), the herbal teas, rooibos (*Aspalathus linearis)* and honeybush (*Cyclopia* spp.), against skin cell viability was investigated *in vitro*.

Methods The effect of extracts, characterised in terms of polyphenol content and antioxidant properties, on cell viability of premalignant, normal and malignant skin cells was determined.

Key findings Phenolic composition, particularly high levels of potent antioxidants, of rooibos and green tea methanol extracts was associated with a strong reduction in cell viability specifically targeting premalignant cells. In contrast, the aqueous extracts of *Cyclopia* spp. were more effective in reducing cell viability. This correlated with a relatively high flavanol/proanthocyanidin content and ABTS radical cation scavenging capacity. The major green tea flavanol (epigallocatechin gallate) and rooibos dihydrochalcone (aspalathin) exhibited differential effects against cell viability, while the major honeybush xanthone (mangiferin) and flavanone (hesperidin) lacked any effect presumably due to a cytoprotective effect. The underlying mechanisms against skin cell viability are likely to involve mitochondrial dysfunction resulting from polyphenol–iron interactions.

Conclusions The polyphenol constituents and antioxidant parameters of herbal tea extracts are useful tools to predict their activity against skin cell survival *in vitro* and potential chemopreventive effects *in vivo*.

Introduction

Polyphenols, present in common dietary sources such as fruit, vegetables and tea, possess potent antioxidant properties that are associated with the prevention of various chronic diseases and cancer.[1] Chemopreventive studies, targeting the reversible stage of cancer promotion, have shown that polyphenols can prevent tumour development in different organs including skin.[2,3] Protection against skin carcinogenesis is achieved either through oral consumption or by topical application with the latter being more effective.[4] Consequently, botanical preparations containing high levels of polyphenols have gained considerable popularity as emerging active ingredients in cosmeceutical formulations.[5] These botanical-derived products have been targeted for use as a novel strategy against the rising morbidity rate of skin cancer. However, the underlying mechanisms involved in their chemopreventive properties in the skin are still unclear.[6,7]

One of the most commonly used plants in cosmeceutical products and also studied extensively as chemopreventive agent in skin is green tea (*Camellia sinensis*).[8,9] Green tea exhibits protective effects against several stages of skin carcinogenesis with the catechins, particularly epigallocatechin

gallate (EGCG) known to be a key role player. Several studies indicate that EGCG prevents tumour development in skin by selectively killing cancer cells through various mechanisms that involve alteration of gene and protein expression, cell cycle signalling pathways, cell metabolism and induction of mitochondrial dysfunction.[10–13] One mechanism by which EGCG may induce mitochondrial dysfunction in epithelial cancer cells is the impairment of respiratory chain complexes, leading to ATP reduction, cell cycle arrest and apoptosis.[14] The chemopreventive properties of green tea polyphenols are generally attributed to their antioxidant or pro-oxidant properties, which are associated with the protection of normal cells and selective killing of cancer cells, respectively.[15,16]

Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) are South African herbal teas that gained interest for use in the treatment of skin disease.[17,18] Incorporation of their extracts into various skin care products relies on anecdotal evidence and thus needs to be supported with sound scientific evidence. The major monomeric polyphenol found in rooibos is the dihydrochalcone, aspalathin, while major honeybush polyphenols are, among others, the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin.[19,20] These polyphenols and herbal tea extracts possess antioxidant properties associated with the prevention of cancer development.[21–25] The chemopreventive properties of rooibos and honeybush extracts have been demonstrated in various organs as well as in the skin.[26–28] The antitumour and photoprotective effects of 'unfermented' (unoxidised) rooibos and honeybush herbal tea extracts on mouse skin cancer models have been reported, but the underlying mechanisms are still not clear.[26, 29]

The aim of this study was to investigate the effect of different rooibos and honeybush extracts and selected polyphenolic constituents on the viability of normal, premalignant and malignant skin cancer cells *in vitro* in relation to their diverse polyphenol composition and antioxidant properties. The current investigation provides the first evidence of the possible underlying mechanisms involved in the disruption of cell viability of human skin cancer cells by the polyphenolic constituents of these herbal teas. The novel role of their polyphenols as tools to predict the cytotoxic activity of the extracts *in vitro* was also evaluated.

Materials and Methods

Chemicals

2,2′-Azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), thiobarbituric acid (TBA), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,4,5-tri (2-pyridyl)-S-triazine (TPTZ), DMSO, gallic acid, (+)-catechin, (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), caffeine, mangiferin and hesperidin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Aspalathin and nothofagin were purified from unfermented rooibos to a purity of >95% by HPLC at the Institute of Biomedical and Microbial Biotechnology, Cape Peninsula University of Technology, Bellville, South Africa. Phenyl pyruvic acid-2-*O*-glucoside (PPAG) and isomangiferin were purified from unfermented rooibos and unfermented *C. subternata*, respectively, to a purity of >95% by the Agricultural Research Council (ARC), Infruitec-Nietvoorbij (Stellenbosch, South Africa). Orientin and isoorientin were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and vitexin, isovitexin, hyperoside, isoquercitrin, luteolin, luteolin-7-*O*-glucoside, rutin and eriocitrin from Extrasynthese (Genay, France). Folin–Ciocalteu reagent, *p*-dimethylaminocinnamaldehyde (DMACA), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and all other analytical reagents used were purchased from Merck (Darmstadt, Germany).

Plant material and extracts

Green tea (*C. sinensis*), imported from China, was a gift from Vital Health Foods (Kuilsriver, South Africa). 'Unfermented' ('green' or 'unoxidised') rooibos (*A. linearis*) was obtained from Rooibos Ltd (Clanwilliam, South Africa), while 'unfermented honeybush' (*C. intermedia*, *C. subternata*, *C. genistoides* and *C. longifolia*) were provided by ARC Infruitec-Nietvoorbij. Aqueous extracts were prepared in triplicate by steeping the plant material (100 g) in freshly boiled deionised water (1000 ml) for 30 min. Extracts were coarse filtered through a double-layer cheese cloth, followed by sequential filtration through Whatman No. 4 and No. 1 filter papers and determination of the soluble solid content whereafter the filtrate was freeze-dried. For preparation of the methanol extracts, the plant material (50 g) was defatted in triplicate by extraction with chloroform (3 × 300 ml) for 24 h. The residual plant material was subsequently extracted with methanol (3 × 300 ml) for 1 h, and the resultant extracts were pooled and dried in vacuo at 40 °C. The extraction yields were determined before pulverisation. All extracts were stored desiccated in amber vials at room temperature until used.

Polyphenol analyses

The total polyphenol (TP) content of the extracts was determined according to the standard method of Singleton and Rossi.[30] Both the methanol and aqueous extracts were reconstituted in deionised H_2O (0.05% m/v) for analysis.

Gallic acid was used as standards and results were expressed as mg gallic acid equivalents/100 mg extract.

Extracts, dissolved in deionised water (0.05% m/v), were reacted with DMACA reagent to quantify the flavanol (FLAVA), including proanthocyanidin content.[31] The reaction was allowed to continue until maximum absorbance was reached at 640 nm. (+)-Catechin, dissolved in methanol was used as a standard and the FLAVA content expressed as mg catechin equivalents/100 mg extract.

Individual polyphenols were quantified by HPLC-DAD analysis, performed on an Agilent 1200 HPLC system, consisting of a quaternary pump, autosampler, inline degasser, column oven and fluorescence and diode array detectors (Agilent Technologies Inc., Santa Clara, USA). Ascorbic acid (final concentration 10 mg/ml) was added to calibration mixtures and samples to protect phenolic compounds from oxidative degradation during preparation and analysis. Authentic standards were used for quantification, except when noted otherwise.

Green tea

HPLC analysis was conducted using a modified version of the method of Lin *et al.*[32] Separation was performed at 30 °C and a flow rate of 1 ml/min on a Gemini C18 column (150 × 4.6 mm, 5 μm particle size, 110 Å pore size) (Phenomenex, Santa Clara, CA, USA), protected by a guard column containing the same stationary phase. Solvent A, 0.1% formic acid (v/v), and solvent B, acetonitrile, were used in the following solvent gradient: 0–6 min (12% B), 6–7 min (12–18% B), 7–14 min (18–25% B), 14–19 min (25–40% B), 19–24 min (40–50% B), 24–29 min (50–12% B) and 29–40 min (12% B). (+)-Catechin and (−)-epicatechin were quantified using fluorescence detection (excitation = 275 nm; emission = 315 nm). (−)-Epigallocatechin gallate (275 nm), (−)-epigallocatechin (275 nm), (−)-epicatechin gallate (275 nm) and caffeine (270 nm) were quantified by UV–vis detection.

Herbal teas

Quantification of aspalathin, nothofagin, PPAG, orientin, isoorientin, vitexin, isovitexin, hyperoside, isoquercitrin, rutin, quercetin-3-*O*-robinobioside and luteolin-7-*O*-glucoside in rooibos extracts was conducted according to the HPLC method described by Beelders *et al.*[20] Aspalathin, nothofagin and PPAG were quantified at 288 nm and the other compounds at 350 nm. Quercetin-3-*O*-robinobioside was quantified as rutin equivalents due to unavailability of an authentic standard. Extracts of *Cyclopia* species (honeybush) were analysed by HPLC as described by de Beer and Joubert and Malherbe *et al.*[19,33] Eriodictyol glucoside, phloretin-3′,5′-di-*C*-glucoside, iriflophenone-3-*C*-glucoside and scolymoside were quantified as eriocitrin, phloretin-3′-*C*-glucoside (nothofagin), hesperidin and luteolin equivalents, respectively. The two xanthones and the flavone, scolymoside, were quantified at 320 nm, while remaining compounds were quantified at 288 nm.

Antioxidant properties

The ABTS radical cation scavenging capacity of the extracts was determined as previously described,[25,34] the ferric reducing antioxidant power according to the method of Benzie and Strain[35] and the oxygen radical antioxidant capacity (ORAC) according to the method of Huang *et al.*[36] For all assays, Trolox was used as standard and activity expressed as mmol Trolox equivalents/g extract. The assays were conducted twice with triplicate determinations for each extract.

Inhibition of iron-induced microsomal lipid peroxidation (LPO) by the extracts was determined according to the method described by Snijman *et al.*[25] Liver microsomes were prepared from male Fischer 344 rats utilising a Sepharose 2B column as described previously by Gelderblom *et al.*[37] IC_{50} values were calculated using the four-parameter logistic curve (Sigmoidal variable slope) in GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, USA). The assay was conducted twice with triplicate determinations for each extract.

Studies in cell cultures

Spontaneously immortalised keratinocytes (HaCaT) were a gift from the Department of Human Biology of the University of Cape Town (South Africa). The HaCaT keratinocyte cell line has various features of premalignant or cancerous cells and it has been utilised as a premalignant cell model.[38–40] Non-malignant normal fibroblast-like skin cells (CRL 7761) and basal carcinoma malignant skin cells (CRL 7762), collected from the same patient, were purchased from the American Tissue Culture Collection (ATCC; Manassas, USA). Premalignant cells (HaCaT) were cultured in RPMI-1640 (Lonza, Walkersville, USA), while Dulbecco's modified Eagle's medium (DMEM; Lonza, Walkersville, USA) was used for the normal and malignant cancer skin cells. The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and L-glutamine (2 mM), while for the malignant skin cancer cells DMEM was also supplemented with HCl to a final concentration of 0.12 mM. Cells were plated in a 96-well microtitre plate at a density of 5×10^3 per well in the respective media (100 μl) containing 10% FBS and cultured for 24 h in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Absence of mycoplasma contamination in the different cell lines was confirmed routinely.

Treatment with extracts and pure compounds

Plant extracts and compounds (EGCG, aspalathin, mangiferin and hesperidin) were dissolved in DMSO and dilutions prepared in the culture media containing 0.5% FBS. All samples were filter sterilised (0.22 μm) and the final DMSO concentration did not exceed 2% for skin cancer cells and 0.5% DMSO for HaCaTs and normal skin cells. Cells were incubated for 24 h before determining cell viability.

Cell viability assay

As plant extracts interfere with the redox chemistry of the routinely used standard LDH and MTT assays,[41] a chemiluminescence assay was used to determine ATP content in the cells following the manufacturer's protocol (CellTiter-Glo®Luminescent cell viability assay from Promega, Madison, USA). The luminescence signal was expressed as relative light units. IC_{50} values were calculated using the 4-parameter logistic curve (Sigmoidal variable slope) in GraphPad Prism. Four to five replicates of the different dilutions of each extract were tested, and the experiment was repeated at least three times.

Statistical analyses

Analysis of variance (ANOVA) was used to test for significant group effects when more than two groups were present, using the generalised linear model procedure (SAS V9.4, Cary, NC, USA). For unbalanced data Tukey–Kramer adjustments were automatically made. Levene's test was used to test for homogeneity of variances and Tukey's test as the post hoc test. Where only two groups were compared, *t*-tests were used. Statistical significance was measured at $P < 0.05$. Spearman's rank correlation coefficients were determined for the honeybush extracts, measuring the strength of the relationship between the different antioxidant, total polyphenol and cell viability variables. Correlation was defined[42] as weak ($r < 0.35$), moderate ($r > 0.35$–0.67) or strong $r > 0.67$) and can be either negative or positive.

Results

Polyphenol analysis

The TP and FLAVA content of the extracts is summarised in Table 1. Methanol extracts had a higher ($P < 0.0001$) TP content than the corresponding aqueous extracts, except for *C. intermedia* extracts having a similar TP content. For the aqueous extracts, rooibos and *C. longifolia* had the highest TP content, while green tea had the lowest TP content. The methanol extract of rooibos and *C. intermedia* had the highest and lowest TP contents, respectively, while green tea had a similar content as *C. longifolia*. Considering the FLAVA (DMACA-reactive substances) content of the extracts, the highest level was found for green tea extracts, in particular its methanol extract. Similarly, the FLAVA content of the rooibos methanol extract was higher than that of its aqueous extract. However, the opposite trend was noticed for the *Cyclopia* species where the aqueous extracts had the highest levels.

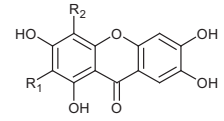
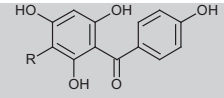
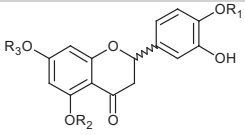
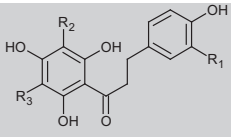
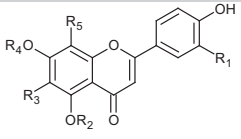
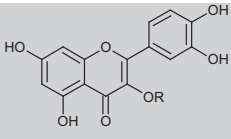
The diverse chemical structures of rooibos and honeybush polyphenols are summarised in Table 2. Methanol extracts of both green tea and rooibos contained significantly higher levels of the individual polyphenols ($P < 0.05$) than their aqueous extracts (Table 3). The major flavanol in the green tea extracts was EGCG, followed by EGC > ECG > EC > (+)-catechin. Substantial levels of caffeine were present in both extracts. The dihydrochalcone, aspalathin, and to a lesser extent its 3-deoxy analogue, nothofagin, were the major polyphenols in both methanol and aqueous extracts of rooibos, followed by the flavone derivatives of aspalathin, with isoorientin > orientin. The other flavones and the flavonols were present in much lower

Table 1 Comparative total polyphenolic and FLAVA content of green tea, rooibos and honeybush herbal tea extracts

		Tea and herbal teas					
Parameter	Extract type*	*Camellia sinensis*	*Aspalathus linearis*	*Cyclopia genistoides*	*Cyclopia longifolia*	*Cyclopia intermedia*	*Cyclopia subternata*
Total polyphenols (mg GAE/100 mg extract)	MeOH	$25.65 \pm 3.27^{b}{}_{A}$	$35.07 \pm 3.44^{a}{}_{A}$	$21.60 \pm 2.43^{c}{}_{A}$	$26.10 \pm 2.40^{b}{}_{A}$	$17.21 \pm 1.82^{d}{}_{A}$	$22.05 \pm 1.51^{c}{}_{A}$
	Aq	$16.10 \pm 2.16^{c}{}_{B}$	$25.05 \pm 2.84^{a}{}_{B}$	$19.39 \pm 1.39^{b}{}_{B}$	$23.95 \pm 2.46^{a}{}_{B}$	$16.45 \pm 2.06^{c}{}_{A}$	$17.50 \pm 2.25^{bc}{}_{B}$
FLAVA (mg CE/100 mg extract)	MeOH	$13.23 \pm 0.37^{a}{}_{A}$	$2.71 \pm 0.16^{b}{}_{A}$	$1.22 \pm 0.16^{d}{}_{B}$	$1.20 \pm 0.16^{d}{}_{B}$	$1.13 \pm 0.10^{d}{}_{B}$	$1.40 \pm 0.15^{c}{}_{B}$
	Aq	$7.76 \pm 0.31^{a}{}_{B}$	$1.80 \pm 0.15^{c}{}_{B}$	$1.62 \pm 0.21^{d}{}_{A}$	$1.45 \pm 0.16^{d}{}_{A}$	$1.79 \pm 0.14^{c}{}_{A}$	$2.25 \pm 0.20^{b}{}_{A}$

Values represent means ± standard deviations of three replications of at least two experiments. Means in a row followed by the same letter (lower case in superscript) or in a column in upper case (for extract type) do not differ significantly; if letters differ, then $P < 0.05$. GAE, gallic acid equivalents; CE, catechin equivalents; FLAVA, flavanols/proanthocyanidins. *MeOH, methanol; Aq, aqueous.

Table 2 Diverse chemical structures of the polyphenolic constituents of rooibos and honeybush herbal teas

Chemical structures and names		RB	HB
	Xanthones		
	Mangiferin: R_1 = β-D-glucopyranosyl; R_2 = H		✓
	Isomangiferin: R_1 = H; R_2 = β-D-glucopyranosyl		✓
	Benzophenone		✓
	Iriflophenone-3-*C*-β-D-glucoside: R = β-D-glucopyranosyl		
	Flavanones		
	Hesperidin: R_1 = CH_3; R_2 = H; R_3 = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl		✓
	Eriocitrin: R_1, R_2 = H; R_3 = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl		✓
	Eriodictyol glucoside[a]: R_1 = H; R_2 or R_3 = β-D-glucopyranosyl (with: R_2 or R_3 = H)*		✓
	Dihydrochalcones		
	Aspalathin: R_1 = OH; R_2 = H; R_3 = β-D-glucopyranosyl	✓	
	Nothofagin: R_1, R_2 = H; R_3 = β-D-glucopyranosyl	✓	
	Phloretin-3′,5′-di-*C*-β-D-glucoside: R_1 = H; R_2, R_3 = β-D-glucopyranosyl		✓
	Flavones		
	Luteolin: R_1 = OH; R_2, R_3, R_4, R_5 = H	✓	✓
	Scolymoside: R_1 = OH; R_2, R_3, R_5 = H; R_4 = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl		✓
	Orientin: R_1 = OH; R_2, R_3, R_4 = H; R_5 = β-D-glucopyranosyl	✓	
	Isoorientin: R_1 = OH; R_2, R_4, R_5 = H; R_3 = β-D-glucopyranosyl	✓	
	Vitexin: R_1, R_2, R_3, R_4 = H; R_5 = β-D-glucopyranosyl	✓	
	Isovitexin: R_1, R_2, R_4, R_5 = H; R_3 = β-D-glucopyranosyl	✓	
	Flavonols		
	Quercetin-3-*O*-robinobioside: R = α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyl	✓	
	Isoquercitrin: R = β-D-glucopyranosyl	✓	
	Rutin: R = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl	✓	
	Hyperoside: R = β-D-galactopyranosyl	✓	

RB = rooibos; HB = honeybush. [a]Tentative identification. *If R2 = β-D-glucopyranoside then R3 = H and vice versa

quantities. As expected, the major honeybush polyphenols in the methanol extracts were the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin (Table 4). *Cyclopia longifolia* and *C. genistoides* extracts had the highest ($P < 0.05$) xanthone levels (xanthone-rich), while *C. intermedia* and *C. subternata* extracts had the highest flavanone levels (flavanone-rich). In the xanthone-rich species, the mean xanthone-to-flavanone ratios varied between approximately 6 : 1 and 8 : 1 in the methanol extracts of *C. genistoides* and *C. longifolia*, respectively. In their aqueous extracts, the respective mean ratios increased to 17 : 1 and 19 : 1. In the flavanone-rich extracts, the xanthone-to-flavanone ratio was approximately 1 : 1 in the methanol extracts, while in the aqueous extracts it was 6 : 1 and 2 : 1 for *C. intermedia* and *C. subternata*, respectively. This substantial change in the xanthone-to-flavanone ratio was due to the significant reduction in the concentration of hesperidin that is poorly soluble in water. Considering the other compounds, *C. genistoides* extracts had the highest iriflophenone-3-*C*-glucoside content, while the *C. subternata* extracts contained the most phloretin-3′,5′-di-*C*-glucoside. Scolymoside, eriocitrin and eriodictyol glucoside were the most abundant in *C. subternata* extract and luteolin in the methanol extract of *C. intermedia.* Eriodictyol glucoside and scolymoside were not detected in the aqueous extract of *C. intermedia.* Scolymoside and eriodictyol glucoside were not detected in *C. genistoides* and *C. longifolia* extracts, respectively.

Antioxidant properties

The ABTS radical cation scavenging capacity of the methanol extracts of rooibos and green tea was significantly ($P < 0.05$) higher when compared to their

Table 3 Individual polyphenol content of extracts prepared from green tea and rooibos as determined by HPLC

Camellia sinensis				*Aspalathus linearis*			
Subgroups	Individual polyphenols	*Methanol* (μg/mg extract)	*Aqueous*	Subgroups	Individual polyphenols	*Methanol* (μg/mg extract)	*Aqueous*
Flavanols	EGCG	$111.93 \pm 3.01_A$	$46.10 \pm 1.49_B$	DHC	Aspalathin	$124.24 \pm 1.44_A$	$83.87 \pm 2.08_B$
	ECG	$20.37 \pm 3.53_A$	$7.48 \pm 4.31_B$		Nothofagin	$27.59 \pm 0.38_A$	$16.68 \pm 0.27_B$
	EGC	$42.25 \pm 1.79_A$	$31.98 \pm 3.20_B$		Total	$151.82 \pm 1.66_A$	$100.55 \pm 1.81_B$
	EC	$14.92 \pm 1.06_A$	$11.28 \pm 1.16_B$	Flavones	Isoorientin	$15.79 \pm 0.10_A$	$10.94 \pm 1.95_B$
	Catechin	$1.32 \pm 0.78_A$	$1.13 \pm 1.15_B$		Orientin	$11.60 \pm 0.06_A$	$8.88 \pm 1.45_B$
	Total	$190.79 \pm 6.23_A$	$97.97 \pm 6.29_B$		Vitexin	$1.60 \pm 0.00_A$	$1.20 \pm 0.00_B$
					Isovitexin	$2.62 \pm 0.02_A$	$1.51 \pm 0.16_B$
Alkaloid	Caffeine	$57.54 \pm 1.59_A$	$40.10 \pm 0.30_B$		Luteolin-7-O-glucoside	$1.78 \pm 0.09_A$	$0.45 \pm 0.11_B$
					Total	$33.39 \pm 0.09_A$	$22.98 \pm 3.47_B$
				Flavonols	Rutin	$4.26 \pm 0.01_A$	$3.60 \pm 0.01_B$
					Hyperoside	$3.53 \pm 0.07_A$	$1.48 \pm 0.77_B$
					Isoquercitrin	$4.51 \pm 0.03_A$	$2.00 \pm 0.89_B$
					QROB	$1.16 \pm 0.00_A$	$0.75 \pm 0.00_B$
					Total	$13.46 \pm 1.53_A$	$7.83 \pm 1.21_B$

Values represent means ± standard deviations of triplicate determinations. Comparison between aqueous and methanolic extracts was analysed using the Student's *t*-test. Means for green tea and rooibos (in a row) followed by the same upper case letters in subscript do not differ significantly; if letters differ, then $P < 0.05$. EGCG, epigallocatechin gallate; EGC, epigallocatechin; ECG, epicatechin gallate; EC, epicatechin; DHC, dihydrochalcones; QROB, quercetin-3-*O*-robinobioside.

aqueous extracts while the opposite effect was noticed for the honeybush species (Table 5). *Cyclopia subternata* exhibited the highest radical scavenging capacity of all the aqueous extracts with green tea having the lowest response.

The ferric iron reducing potential of the methanol extracts of rooibos and green tea exhibited similar capacity and was significantly higher ($P < 0.05$) than their aqueous extracts. Of the honeybush methanol extracts, the xanthone-rich species, *C. genistoides* and *C. longifolia,* exhibited higher ($P < 0.05$) capacity than the flavanone-rich *C. intermedia* and *C. subternata*. For the aqueous extracts only *C. longifolia* exhibited a significant ($P<0.05$) higher capacity. The aqueous extracts of *C. intermedia* and *C. subternata* were more active than their corresponding methanol extracts while no significant difference ($P > 0.05$) was found between the methanol and aqueous extracts of *C. genistoides* and *C. longifolia*.

In the ORAC assay, contrary to the other antioxidant assays, the herbal teas exhibited higher antioxidant capacity than green tea with rooibos being the most active. For both the methanol and aqueous extracts, the descending order of capacity was rooibos > *C. longifolia* > *C. subternata* ≅ *C. genistoides* > *C. intermedia* > green tea with the methanol extracts being the more active.

For inhibition of iron-induced LPO, all the methanol extracts exhibited lower IC_{50} values, indicating a higher ($P < 0.05$) protective effect than their respective aqueous extracts. Green tea and rooibos extracts had similar capacity. Overall, *C. intermedia* extracts were the least effective in inhibiting iron-induced LPO.

Modulation of cell viability (cellular ATP content)

Methanol extracts of green tea were more active ($P < 0.05$) than their corresponding aqueous extracts in disrupting cell viability in the different skin cell cultures (Table 6). For rooibos extracts, the methanol extract exhibited higher activity than the aqueous extract in the normal cells. A similar trend, although not significant ($P \geq 0.05$), was also observed for rooibos extracts in the premalignant cell (HaCaT) line while, in the malignant cells, the aqueous extract was significantly more active than the methanol extract. The premalignant cells were the most sensitive cell type to the effects of both green tea and rooibos extracts.

In contrast, the aqueous extracts of the different honeybush species exhibited a higher activity against the viability of the normal and malignant cancer cells than the methanol extracts. However, in the premalignant cells, there was no significant difference between the types of extract, except for aqueous extract of *C. longifolia* displaying higher activity than its methanol extract. The premalignant cells were also the most sensitive ($P < 0.05$) cell type to the methanol extracts of the honeybush species. A similar effect was noticed for the aqueous extract of *C. genistoides*. The methanol extract of *C. longifolia* exhibited a higher sensitivity ($P < 0.05$) to malignant cells when compared to the normal cell line.

Comparisons between the extracts of the different plants indicated that rooibos extracts were the most active of the herbal teas against all three cell lines with its

Table 4 Concentration of individual polyphenols (μg/mg extract) prepared from different honeybush species as determined by HPLC

		Cyclopia genistoides				*Cyclopia longifolia*			
Cyclopia spp. (Xanthone-rich)	Polyphenols	Methanol	Xan/Fla Ratio	Aqueous	Xan/Fla Ratio	Methanol	Xan/Fla Ratio	Aqueous	Xan/Fla Ratio
Xanthone	Mangiferin	$156.07 \pm 4.31_A$	6:1	$77.48 \pm 0.25_B$	17:1	$180.04 \pm 6.18_A$	8:1	$126.99 \pm 3.36_B$	19:1
	Isomangiferin	$39.99 \pm 0.93_A$		$30.11 \pm 0.84_B$		$48.99 \pm 1.94_A$		$33.09 \pm 0.78_B$	
	Total	$196.06 \pm 4.66_A$		$107.59 \pm 0.65_B$		$229.04 \pm 5.61_A$		$160.08 \pm 4.11_B$	
Flavanone	Eriocitrin	$1.61 \pm 0.00_A$		$1.11 \pm 0.03_B$		$2.83 \pm 0.09_A$		$1.82 \pm 0.08_B$	
	Hesperidin	$32.94 \pm 1.03_A$		$4.12 \pm 0.10_B$		$24.60 \pm 0.65_A$		$6.73 \pm 0.38_B$	
	Eriodictyol glucoside	$1.13 \pm 0.02_A$		$1.15 \pm 0.02_A$		–		–	
	Total	$35.67 \pm 1.02_A$		$6.38 \pm 0.10_B$		$27.43 \pm 0.73_A$		$8.54 \pm 0.46_B$	
Flavone	Luteolin	$0.84 \pm 0.02_A$		$0.16 \pm 0.01_B$		$0.77 \pm 0.02_A$		$0.17 \pm 0.01_B$	
	Scolymoside	–nd		–		$4.30 \pm 0.10_A$		$3.30 \pm 0.11_B$	
	Total	$0.84 \pm 0.02_A$		$0.16 \pm 0.01_B$		$5.07 \pm 0.11_A$		$3.47 \pm 0.12_B$	
DHC	Phloretin-3′,5′-di-C-glucoside	$2.54 \pm 0.08_A$		$2.30 \pm 0.02_B$		$1.31 \pm 0.25_A$		$0.83 \pm 0.08_B$	
Benzophenone	Iriflophenone-3-C-glucoside	$20.76 \pm 0.35_A$		$16.95 \pm 0.25_B$		$12.55 \pm 0.98_A$		$8.81 \pm 0.61_B$	
Cyclopia spp. (Flavanone-rich)	Polyphenols	*Cyclopia intermedia*				*Cyclopia subternata*			
Xanthone	Mangiferin	$67.70 \pm 2.16_A$	1:1	$39.77 \pm 0.41_B$	6:1	$62.57 \pm 1.46_A$	1:1	$22.02 \pm 2.99_B$	2:1
	Isomangiferin	$20.02 \pm 0.71_A$		$14.26 \pm 0.40_B$		$15.95 \pm 0.26_A$		$8.70 \pm 1.46_B$	
	Total	$87.77 \pm 2.87_A$		$54.04 \pm 0.75_B$		$78.52 \pm 1.26_A$		$30.72 \pm 4.45_B$	
Flavanone	Eriocitrin	$2.93 \pm 0.20_A$		$1.25 \pm 0.06_B$		$5.25 \pm 0.25_A$		$3.27 \pm 0.25_B$	
	Hesperidin	$88.77 \pm 11.55_A$		$7.32 \pm 0.56_B$		$63.15 \pm 8.62_A$		$7.98 \pm 0.21_B$	
	Eriodictyol glucoside	0.39 ± 0.00A		–		$3.14 \pm 0.21_B$		$3.87 \pm 0.20_A$	
	Total	$92.10 \pm 11.74_A$		$8.57 \pm 0.62_B$		$71.54 \pm 9.07_A$		$15.12 \pm 0.23_B$	
Flavone	Luteolin	$3.62 \pm 0.27_A$		$0.23 \pm 0.01_B$		$1.10 \pm 0.06_A$		$0.13 \pm 0.05_B$	
	Scolymoside	0.96 ± 0.14		–		$9.16 \pm 0.31_A$		$4.03 \pm 0.13_B$	
	Total	$4.58 \pm 0.26_A$		$0.23 \pm 0.01_B$		$10.26 \pm 0.37_A$		$4.16 \pm 0.09_B$	
Dihydrochalcone	Phloretin-3′,5′-di-C-glucoside	$0.65 \pm 0.03_A$		$0.68 \pm 0.01_A$		$14.13 \pm 0.88_A$		$12.53 \pm 1.42_B$	
Benzophenone	Iriflophenone-3-C-glucoside	$3.91 \pm 0.27_A$		$3.63 \pm 0.11_A$		$13.69 \pm 0.35_A$		$9.32 \pm 0.16_B$	

Values represent means ± standard deviations of triplicate determinations. Comparison between aqueous and methanol extracts was analysed by Student's *t*-test and significant differences are indicated if $P < 0.05$. Means for each tea or herbal tea (in a row) followed by the same upper case letters in subscript do not differ significantly; if letters differ, then $P < 0.05$. DHC, dihydrochalcones; Xan/Fla ratio, xanthone-to-flavanone ratio. nd, not detected.

Table 5 Comparative antioxidant capacity of green tea, rooibos and honeybush herbal tea extracts utilising different antioxidant assays

		Tea and herbal teas					
Antioxidant assay	Extract type*	*Camellia sinensis*	*Aspalathus linearis*	*Cyclopia genistoides*	*Cyclopia longifolia*	*Cyclopia intermedia*	*Cyclopia subternata*
ABTS	MeOH	$10.90 \pm 0.75^{b}_{A}$	$11.49 \pm 0.81^{a}_{A}$	$8.08 \pm 0.21^{d}_{B}$	$8.41 \pm 0.42^{cd}_{B}$	$6.68 \pm 0.29^{e}_{B}$	$8.70 \pm 0.31^{c}_{B}$
(mmol TE/g	Aq	$6.77 \pm 0.65^{d}_{B}$	$9.08 \pm 0.45^{b}_{B}$	$8.87 \pm 0.54^{b}_{A}$	$9.37 \pm 0.33^{b}_{A}$	$8.12 \pm 0.20^{c}_{A}$	$10.09 \pm 0.39^{a}_{A}$
extract)							
FRAP	MeOH	$3.08 \pm 0.34^{a}_{A}$	$3.04 \pm 0.19^{a}_{A}$	$1.77 \pm 0.16^{c}_{A}$	$2.01 \pm 0.14^{b}_{A}$	$1.30 \pm 0.07^{e}_{B}$	$1.56 \pm 0.08^{d}_{B}$
(mmol TE/g	Aq	$1.81 \pm 0.14^{bc}_{B}$	$2.24 \pm 0.18^{a}_{B}$	$1.68 \pm 0.16^{cd}_{A}$	$1.88 \pm 0.23^{b}_{A}$	$1.61 \pm 0.11^{d}_{A}$	$1.67 \pm 0.11^{cd}_{A}$
extract)							
ORAC	MeOH	$7.77 \pm 0.09^{e}_{A}$	$14.02 \pm 1.01^{a}_{A}$	$10.46 \pm 0.75^{c}_{A}$	$11.91 \pm 0.38^{b}_{A}$	$8.92 \pm 0.22^{d}_{A}$	$10.53 \pm 0.57^{c}_{A}$
(mmol TE/g	Aq	$4.37 \pm 0.10^{e}_{B}$	$9.12 \pm 0.53^{a}_{B}$	$7.22 \pm 0.32^{c}_{B}$	$8.55 \pm 0.09^{b}_{B}$	$6.57 \pm 0.29^{d}_{B}$	$7.27 \pm 0.23^{c}_{B}$
extract)							
LPO	MeOH	$0.23 \pm 0.01^{c}_{B}$	$0.24 \pm 0.01^{c}_{B}$	$0.79 \pm 0.01^{a}_{A}$	$0.75 \pm 0.01^{ab}_{B}$	$0.82 \pm 0.01^{a}_{B}$	$0.66 \pm 0.005^{b}_{B}$
(IC_{50} – mg/ml)	Aq	$0.34 \pm 0.01^{c}_{A}$	$0.33 \pm 0.00^{c}_{A}$	$0.89 \pm 0.02^{b}_{A}$	$0.98 \pm 0.01^{ab}_{A}$	$1.14 \pm 0.01^{a}_{A}$	$0.81 \pm 0.011^{b}_{A}$

Values represent means ± standard deviations of three to five replications of at least two experiments. Means in a row (tea and herbal teas) followed by the same letter (lower case in superscript) or in upper case in a column (extract type per assay) do not differ significantly; if letters differ, then $P < 0.05$. TE, Trolox equivalents; FRAP, ferric reducing antioxidant potential; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid); LPO, lipid peroxidation; ORAC, oxygen radical scavenging capacity. *MeOH, methanol; Aq, aqueous.

Table 6 Comparative IC_{50} concentrations of different extracts of green tea, rooibos and honeybush herbal teas on the cell viability of the different skin cells

		Tea and herbal teas					
IC_{50} (Cell Viability) (mg/ml)	Extract type*	*Camellia sinensis*	*Aspalathus linearis*	*Cyclopia genistoides*	*Cyclopia longifolia*	*Cyclopia intermedia*	*Cyclopia subternata*
Premalignant	MeOH	$0.08 \pm 0.01^{c}_{B}$	$0.13 \pm 0.02^{c}_{A}$	$\mathbf{0.72 \pm 0.10^{a}_{A}}$	$\underline{0.72 \pm 0.10^{a}_{A}}$	$\underline{0.53 \pm 0.07^{b}_{A}}$	$\mathbf{0.47 \pm 0.06^{b}_{A}}$
cells**	Aq	$0.17 \pm 0.03^{d}_{A}$	$0.15 \pm 0.02^{d}_{A}$	$\mathbf{0.68 \pm 0.11^{a}_{A}}$	$0.51 \pm 0.08^{b}_{B}$	$0.48 \pm 0.08^{bc}_{A}$	$0.41 \pm 0.09^{c}_{A}$
Normal	MeOH	$0.23 \pm 0.06^{d}_{B}$	$0.26 \pm 0.05^{d}_{B}$	$1.85 \pm 0.17^{a}_{A}$	$1.14 \pm 0.13^{c}_{A}$	$1.37 \pm 0.17^{b}_{A}$	$1.08 \pm 0.20^{c}_{A}$
cells**	Aq	$0.34 \pm 0.06^{c}_{A}$	$0.29 \pm 0.05^{c}_{A}$	$0.88 \pm 0.19^{a}_{B}$	$0.53 \pm 0.04^{b}_{B}$	$0.50 \pm 0.13^{b}_{B}$	$0.37 \pm 0.07^{c}_{B}$
Malignant	MeOH	$0.21 \pm 0.06^{d}_{B}$	$0.31 \pm 0.05^{d}_{A}$	$2.29 \pm 0.61^{a}_{A}$	$\underline{0.80 \pm 0.16^{c}_{A}}$	$1.29 \pm 0.23^{b}_{A}$	$1.14 \pm 0.15^{b}_{A}$
cells**	Aq	$0.41 \pm 0.07^{c}_{A}$	$0.26 \pm 0.03^{d}_{B}$	$0.94 \pm 0.24^{a}_{B}$	$0.52 \pm 0.09^{b}_{B}$	$0.44 \pm 0.10^{bc}_{B}$	$0.43 \pm 0.14^{bc}_{B}$

Values represent means ± standard deviations of five replications of at least two experiments. Means in a row (tea and herbal teas) followed by the same letter (lower case in superscript) or in a column in upper case (extract type within each cell type) do not differ significantly; if letters differ, then $P < 0.05$. IC_{50}, IC_{50} values in bold of MeOH extracts and aqueous extract of *C. genistoides* differ significantly (P<0.05) from other cell types; Underlined IC_{50} value differs significantly (P<0.05 from the normal cell counterpart tea/herbal tea concentration yielding 50% inhibition of cell viability; ATP, adenosine triphosphate. *MeOH, methanol; Aq, aqueous. **Premalignant cells, HaCaT keratinocytes; normal cells, CRL 7761; cancer cells, CRL 7762.

activity similar to green tea extracts, except in the malignant cancer cell line where the rooibos aqueous extract was more active. Considering the honeybush species, aqueous extracts of *C. subternata* and *C. intermedia* were the most active. It is evident that the relative activity of the type of honeybush extracts is depended on the cell type. For instance, in the premalignant cell line, the activity of both extracts decreased in the following order: *C. subternata* ≅ *C. intermedia* > *C. longifolia* ≅ *C. genistoides*. For normal cells, the order of activity of the methanol extracts was *C. subternata* ≅ *C. longifolia* > *C. intermedia* > *C. genistoides, and* for the aqueous extracts, it was *C. subternata* > *C. intermedia* ≅ *C. longifolia* > *C. genistoides.* In the malignant cancer cells, the order for the activity of the methanol extracts was *C. longifolia* > *C. subternata* ≅ *C. intermedia* > *C. genistoides,* while for the aqueous extracts it was *C. subternata* ≥ *C. intermedia* ≥ *C. longifolia* > *C. genistoides.* Irrespective of the cell type or honeybush extract tested, *C. genistoides* exhibited the lowest activity against cell viability.

Interrelationships between chemical composition, antioxidant capacity and cell viability

Green tea

The higher TP and FLAVA content (Table 1) of the methanol extract compared with the aqueous extract coincided with a higher antioxidant capacity in the ABTS, FRAP, ORAC and LPO assays (Table 5), as well as a significant reduction (almost twofold) in cell viability (Tables 6).

Table 7 Correlations between polyphenol parameters, antioxidant capacities and modulation of cell viability of methanol and aqueous extracts of honeybush species

Phenolic/antioxidant parameters	FLAVA	ABTS	FRAP	LPO	ORAC	Cell[a] viability (ATP)		
						Premalignant cells	Normal cells	Malignant cells
TP	−0.334 (0.0041)	0.205 (0.0041)	0.517 (0.0001)	−0.412 (0.0037)	0.544 (0.0001)	0.298 (0.0011)	0.208 (0.0121)	Ns
FLAVA	1.000	0.504 (0.0001)	ns	0.365 (0.0105)	−0.775 (0.0001)	−0.754 (0.0001)	−0.539 (0.0001)	−0.538 (0.0001)
ABTS	–	1.000	0.332 (0.0001)	ns	ns	−0.254 (0.0058)	−0.641 (0.0001)	−0.499 (0.0001)
LPO	–	–	–	1.000	−0.672 (0.0001)	NC	−0.317 (0.0382)	−0.396 (0.0053)
ORAC	–	–	–	–	1.000	0.421 (0.0003)	0.628 (0.0001)	0.472 (0.0001)
FRAP	ns	–	1.000	NC	NC	−0.299 (0.0010)	ns	−0.198 (0.0172)

Spearman's correlations were used to calculate correlation coefficients (*r* values). $P < 0.05$ was considered statistically significant (*P*-values given in parentheses). TP, total polyphenols; FLAVA, flavanol/proanthocyanidins; FRAP, ferric reducing antioxidant potential; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid); LPO, lipid peroxidation; ORAC, oxygen radical scavenging capacity; ns, no significant correlation; NC, no correlation; ATP, adenosine triphosphate. [a]Premalignant cells, HaCaT keratinocytes; normal cells, CRL 7761; malignant cells, CRL 7762.

Rooibos

A similar pattern to green tea was observed for rooibos as the higher TP content of the methanol extract coincided with high antioxidant capacity and reduction in cell viability. Higher levels of the dihydrochalcones, flavones and flavonols were present in the methanol extract compared with the aqueous extract (Table 3). Although statistical differences ($P < 0.05$) were noticed between the aqueous and methanol extracts for their effect on the viability of normal and malignant cancer cells, the margin of difference was small (Table 6). Of interest is that the aqueous extract was more active in the malignant cancer cells and the methanol extract more active in the normal cells. However, no significant ($P \geq 0.05$) difference was noticed between the two types of rooibos extract in premalignant cells. No clear association between antioxidant capacity, TP content and FLAVA content of extracts and reduction in cell viability was noticed.

Honeybush

Correlation between TP content, FLAVA content and various indicators of antioxidant capacity of honeybush extracts, varied from weak to moderate (Table 7). TP content of the extracts correlated moderately with activity in the FRAP and ORAC assays, weakly (negative) with FLAVA content and inhibition of LPO and weakly with ABTS radical cation scavenging capacity. In contrast to the TP content, the FLAVA content showed a moderate to weak correlation with ABTS radical cation scavenging capacity, while a strong, but negative correlation with ORAC existed. The correlation with iron-related antioxidant assays was either weak (LPO) or absent (FRAP).

The activity of extracts against cell viability showed a negative correlation with the FLAVA content (moderate to strong, depending on the cell type), ABTS (weak to strong, depending on the cell type) and LPO (moderate for normal and malignant cancer cells) parameters. A positive correlation between cell viability and ORAC depended on the cell type and varied from strong (normal cells) to moderate in premalignant and malignant cancer cells. A weak positive correlation between TP and cell viability was noticed against HaCaT and normal cells.

Activity of selected polyphenolic compounds and relative levels associated with the reduction in cell viability

Of the pure polyphenolic compounds tested, the activity of EGCG was up to 10-fold more ($P < 0.05$) than aspalathin (Table 8). As observed for green tea and rooibos extracts, premalignant cells were the most sensitive cell line. The

malignant cell line showed an increased sensitivity to EGCG compared with normal cells, whereas both cell lines showed similar sensitivity to aspalathin. When considering the IC_{50} values for pure EGCG and aspalathin, their corresponding levels in the respective methanol and aqueous extracts of green tea and rooibos at their IC_{50} for cell viability were associated with much lower (Table 8). Due to the poor solubility of mangiferin and hesperidin in the cell medium, no IC_{50} values could be obtained for these compounds. However, considering the mangiferin, isomangiferin and hesperidin content of the honeybush methanol and aqueous extracts, the latter, containing less of these compounds, exhibited higher activity against cell viability.

Discussion

Carcinogenesis studies in mouse skin indicated that rooibos and honeybush herbal tea extracts exhibit chemopreventive properties by reducing the size and number of tumours.[26,29] Although polyphenolic compounds and antioxidant properties have been implicated in the anticancer properties of the extracts, the underlying mechanisms involved are unclear. To further clarify the relationship between the chemical and biological characteristics of these herbal teas, the present study evaluated the relationship between the polyphenolic content and antioxidant properties and the ability of their extracts to reduce viability of normal, premalignant and malignant cancer skin cells *in vitro*. The distinct differences in phenolic composition and antioxidant capacities of the herbal extracts served to formulate a hypothesis on the possible mechanisms involved in the reduction in skin cell viability. Green tea extracts were used as benchmark.

Rooibos extracts, displaying the highest total polyphenol content and antioxidant capacities, were the most active in reducing skin cell viability, while selectively targeting the growth of premalignant cancer cells. The methanol extract of rooibos, containing higher levels of polyphenols, was

Table 8 Disruption of cell viability in the different skin cells by pure polyphenolic compounds and their corresponding levels in green tea and rooibos extracts at their respective IC_{50} values

Major pure polyphenols	IC_{50} (μg/ml) for cell viability		
	Premalignant cells	Normal cells	Malignant cells
EGCG	$39.2 \pm 5.0^{a}_{A}$ (85.5 ± 10.9 μM)*	$79.4 \pm 5.0^{b}_{A}$ (173.2 ± 10.9 μM)	$59.1 \pm 4.9^{c}_{A}$ (128.7 ± 10.7 μM)
Aspalathin	$230.3 \pm 45.1^{a}_{B}$ (509.1 ± 99.7 μM)	$385.6 \pm 132.7^{b}_{B}$ (852.3 ± 293.3 μM)	$419.1 \pm 114.6^{b}_{B}$ (926.4 ± 252.2 μM)
Mangiferin	>300 (>710.3 μM)	>300 (>710.3 μM)	>300 (>710.3 μM)
Hesperidin	>436 (>1445.7 μM)	>436 (>1445.7 μM)	>436 (>1445.7 μM)

Tea extracts	Major monomeric polyphenols	Monomeric polyphenol levels (μg/ml) associated with IC_{50} for cell viability for each tea/herbal tea extract					
		Premalignant cells		Normal cells		Malignant cells	
		MeOH	Aq	MeOH	Aq	MeOH	Aq
C. sinensis	EGCG	$8.6 \pm 1.3_{A}$	$8.0 \pm 1.6_{A}$	$25.7 \pm 5.0_{A}$	$15.8 \pm 2.3_{B}$	$23.4 \pm 3.8_{A}$	$19.1 \pm 3.9_{B}$
A. linearis	Aspalathin	$16.3 \pm 2.7_{A}$	$11.4 \pm 0.9_{B}$	$31.8 \pm 7.0_{A}$	$24.7 \pm 5.4_{B}$	$38.5 \pm 7.6_{A}$	$22.12 \pm 5.8_{B}$
C. genistoides							
Xanthone	Mangiferin	$112.1 \pm 16.0_{A}$	$52.6 \pm 8.7_{A}$	$288.8 \pm 26.4_{A}$	$68.0 \pm 14.5_{B}$	$357.1 \pm 96.1_{A}$	$72.8 \pm 19.0_{B}$
	Isomangiferin	$28.7 \pm 4.1_{A}$	$20.4 \pm 3.4_{B}$	$74.0 \pm 6.8_{A}$	$26.4 \pm 5.6_{B}$	$91.5 \pm 24.6_{A}$	$28.3 \pm 7.4_{B}$
Flavanone	Hesperidin	$23.7 \pm 3.4_{A}$	$2.8 \pm 0.5_{B}$	$61.0 \pm 5.6_{A}$	$3.6 \pm 0.8_{B}$	$75.4 \pm 20.3_{A}$	$3.9 \pm 1.0_{B}$
C. longifolia							
Xanthone	Mangiferin	$128.9 \pm 18.7_{A}$	$64.6 \pm 11.7_{B}$	$205.4 \pm 22.7_{A}$	$68.0 \pm 5.3_{B}$	$143.2 \pm 28.6_{A}$	$66.1 \pm 14.0_{B}$
	Isomangiferin	$35.1 \pm 5.09_{A}$	$16.8 \pm 3.1_{B}$	$55.9 \pm 6.2_{A}$	$17.7 \pm 1.4_{B}$	$39.0 \pm 7.8_{A}$	$17.2 \pm 3.7_{B}$
Flavanone	Hesperidin	$17.6 \pm 2.6_{A}$	$3.4 \pm 0.6_{B}$	$28.1 \pm 3.1_{A}$	$3.6 \pm 0.3_{B}$	$19.6 \pm 3.9_{A}$	$3.5 \pm 0.7_{B}$
C. intermedia							
Xanthone	Mangiferin	$35.9 \pm 5.0_{A}$	$19.0 \pm 3.3_{B}$	$92.8 \pm 11.5_{A}$	$20.1 \pm 5.3_{B}$	$87.1 \pm 15.5_{A}$	$17.6 \pm 3.9_{B}$
	Isomangiferin	$10.6 \pm 1.5_{A}$	$6.8 \pm 1.2_{B}$	$27.4 \pm 3.4_{A}$	$7.20 \pm 1.9_{B}$	$25.7 \pm 4.6_{A}$	$6.3 \pm 1.4_{B}$
Flavanone	Hesperidin	$47.1 \pm 6.0_{A}$	$3.5 \pm 0.6_{B}$	$121.6 \pm 15.0_{A}$	$3.7 \pm 1.0_{B}$	$114.1 \pm 20.3_{A}$	$3.3 \pm 0.7_{B}$
C. subternata							
Xanthone	Mangiferin	$29.4 \pm 3.9_{A}$	$9.2 \pm 1.9_{B}$	$67.6 \pm 12.3_{A}$	$8.13 \pm 1.6_{B}$	$71.2 \pm 9.5_{A}$	$9.6 \pm 3.1_{B}$
	Isomangiferin	$7.5 \pm 1.0_{A}$	$3.6 \pm 0.8_{B}$	$17.2 \pm 3.1_{A}$	$3.2 \pm 0.6_{B}$	$18.2 \pm 2.4_{A}$	$3.8 \pm 1.2_{B}$
Flavanone	Hesperidin	$29.7 \pm 4.0_{A}$	$3.3 \pm 0.7_{B}$	$68.2 \pm 12.4_{A}$	$3.0 \pm 0.6_{B}$	$71.0 \pm 9.8_{A}$	$3.5 \pm 1.1_{B}$

Values represent means ± standard deviations of triplicate determinations. Means in a row followed by the same letter (lower case superscript) or in a column (upper case subscript) do not differ significantly; if letters differ, then $P < 0.05$. Abbreviations: IC_{50}, tea/herbal tea concentration yielding 50% inhibition of cell viability (ATP content). MeOH, methanol; Aq, aqueous; EGCG, epigallocatechin gallate. Premalignant cells, HaCaT keratinocytes; normal cells, CRL 7761; malignant cells, CRL 7762. *Values in brackets depicted the equivalent micromolar concentrations.

slightly more active than its aqueous extract in normal and premalignant cells, but not against the malignant cells. This suggested that rooibos polyphenols are likely to play an important role during the early stages of cancer development by targeting the viability of precancerous cells. This was further corroborated by the strong activity of aspalathin, the major flavonoid of rooibos, against premalignant cells. However, the relatively high concentration (IC_{50}) of aspalathin required to reduce cell viability compared with its corresponding level associated with the IC_{50} of the extract suggests that it may not be the main active polyphenolic constituent in rooibos extract. In this regard, crude polymeric fractions from rooibos extracts have been reported to be more effective in a lipid environment than fractions enriched in monomeric rooibos compounds.[22] Therefore, it would appear that synergistic or additive effect exists between the different rooibos monomeric and polymeric tannin-like FLAVA constituents against skin cell viability. This would also explain the marginal difference in activity between methanol and aqueous rooibos extracts in cells.

Rooibos extracts exhibited similar trends to the green tea extracts with respect to the TP content, antioxidant capacity (FRAP, LPO and ABTS) and reduction in skin cell viability, indicating possible similarity in their mechanism of action. In this regard, EGCG is known to selectively affect the growth of epithelial cancer cells by decreasing mitochondrial respiratory chain protein level and activity via STAT3 leading to reduction in cellular ATP, cell cycle arrest and apoptosis.[14] Thus, the reduction in skin cell viability via ATP inhibition by aspalathin and other rooibos flavonoids as demonstrated in this study may also involve a similar mechanism although autoxidation may also play a role, as described for EGCG.[14] It is also important to note that studies indicated that other rooibos flavonoids that include orientin, isoorientin, vitexin and luteolin are able to modulate the respiratory chain complexes in cells causing mitochondrial dysfunction that leads to depletion of cellular ATP levels and cell death.[43–46] Another possible mechanism is likely to involve polyphenol–iron interactions given the close association between increased antioxidant capacity of rooibos extracts in iron-related (FRAP; LPO) assays and in the reduction in cell viability. In this regard, desferoxamine, a known iron chelator, was found to induce mitochondrial dysfunction by causing defects in the mitochondrial respiratory chain via downregulation of Fe–sulfur subunits.[47] Thus, as rooibos extracts exhibit iron-chelating activity,[25] a similar effect could prevail in skin cells. The iron-associated pro-oxidant properties of monomeric and polymeric phenolic fractions of rooibos have been demonstrated *in vitro* in a Fenton-type reaction.[22] A specific role of such an interaction was also implicated in the hepatotoxic activity of rooibos extracts and the *in-vivo* protection against liver carcinogenesis.[27]

The extracts of the four *Cyclopia* spp. varied with respect to their phenolic composition, antioxidant capacities and reduction in cell viability. In contrast to rooibos, the aqueous extracts of honeybush, exhibiting higher levels of FLAVA and strong radical scavenging capacity (ABTS), were more active against skin cell viability compared with the methanol extracts. The aqueous extracts of the flavanone-rich *Cyclopia* spp., *C. subternata and C. intermedia*, exhibiting the highest FLAVA content, were the most active in reducing skin cell viability. *Cyclopia subternata* exhibited the highest radical scavenging capacity in the ABTS assay and this corroborated the significant negative correlation that exists between FLAVA, ABTS and reduction in cell viability. However, it would appear that the activity of extracts in cells may be dependent not only on their FLAVA content, but also on a specific xanthone-to-flavanone ratio. Extracts with a low ratio (Table 4), as in the case of *C. subternata* (2 : 1) and *C. intermedia* (6 : 1), were associated with an increased activity against cell viability. It would appear that reduction in cell viability by honeybush species is also dependent on other polyphenolic constituents in the extract. In this regard, the interactive role of other polyphenolic constituents of honeybush including scolymoside, a rutinoside of luteolin, the benzophenone, iriflophenone-3-*C*-glucoside and the dihydrochalcone, phloretin-3′,5′-di-*C*-glucoside is of interest.

Contrary to rooibos and green tea, the role of the major monomeric honeybush polyphenols in the activity of the extracts against skin cell viability is more obscure, given that the methanol extracts, containing higher levels of polyphenols than aqueous extracts, exhibited weaker activity. This is also evident through the very weak correlation between TP content and reduction in cell viability, as well as the lack of any measurable activity for mangiferin and hesperidin. The levels of these compounds associated with the IC_{50} for the aqueous extracts were also far lower compared with the methanol extracts, suggesting a minor role, if any, in the reduction in cell viability. Major polyphenols present in the methanol extracts were the xanthones and flavanones with the xanthone-rich extracts of *C. genistoides* also exhibiting the weakest activity against cell viability, irrespective of cell type. It is possible that the monomeric polyphenols of honeybush is conferring a cytoprotective effect that may be dependent on a specific level or ratio between mangiferin and hesperidin.

When considering the antioxidant properties of the honeybush extracts, the weak correlation between their capacity in the iron-related antioxidant assays (FRAP and LPO) and reduction in cell viability suggests the role of

polyphenol–iron antioxidant mechanism in the cytoprotective effect by these extracts. For instance, the methanol extracts of the xanthone-rich species, specifically *C. genistoides,* tended to exhibit strong antioxidant capacity in the FRAP and LPO assays, but displayed weak activity against cell viability. In this regard, the cytoprotective activity of mangiferin and hesperidin has been demonstrated *in vitro* and it was associated with reduced metal-induced oxidative stress and apoptosis in HepG2 cells and primary hepatocytes, respectively.[48,49] Both mangiferin and hesperidin exhibit a high redox potential that confers a lower electron-donating activity, and therefore, they are not as reactive as catechins and quercetin in the presence of an iron catalyst.[50] It has been suggested that the high redox potential of mangiferin, specifically when in coordination with iron (III), prevents pro-oxidation and mitochondrial dysfunction. The iron-chelating activity of mangiferin in lipid membranes has also been reported to involve a dual mechanism that results in the stimulation of iron (II) autoxidation and the formation of a stable complex with iron (III), known to inhibit mitochondrial lipid peroxidation by scavenging reactive oxygen species.[51,52] Thus, the presence of transition metals in the culture medium or in the cells may stabilise mangiferin and hesperidin, allowing them to exert antioxidant effects against formation of reactive oxygen species. In addition, mangiferin protects cells against the toxic effects of oxidative stress via modulation of nuclear factor erythroid 2-related factor 2 (nrf2) that regulates the expression of antioxidant proteins.[53] A similar protective mechanism that would explain the weaker effect of the methanol extracts against skin cell viability is therefore likely to prevail, but this needs to be investigated further.

Apart from mangiferin and hesperidin, other polyphenols are also likely to be involved in the cytoprotective effects of honeybush. In this regard, the flavanone-rich species, *C. subternata*, exhibiting the highest capacity against lipid peroxidation, contained relatively high levels of eriocitrin, a flavanone suggested to have similar activity to mangiferin in the protection of lipid membranes against oxidative damage.[24] In the present study, the strong protective activity of *C. subternata* and *C. longifolia* against lipid peroxidation coincided with high levels of scolymoside, a rutinoside of luteolin. The latter flavone aglycone is known to protect cells from oxidative stress through its strong radical scavenging activity and free radical stabilisation.[54] Eriodictyol, another honeybush polyphenol forming the flavanone aglycone of eriodictyol glucoside and eriocitrin, has been reported to be very effective in protecting retinal cells against Fe^{2+}-induced oxidative stress.[55] Thus, the major monomeric compounds could be acting synergistically with other minor polyphenolic constituents such as eriocitrin, scolymoside and eriodictyol glucoside in protecting cells by preventing damage of lipid membranes. Comprehensive analysis of the phenolic fraction of *C. genistoides* also revealed the presence of other xanthones, flavanones and benzophenones[56] that could also play a contributing role that should be considered in the future.

Conclusions

The reduction in skin cell viability by rooibos extracts, primarily targeting premalignant cells, seems to be caused by its monomeric polyphenolic compounds while polymeric flavanol-like polyphenolic compounds of honeybush are more reactive. The polyphenolic compounds of rooibos may be useful biomarkers to predict the cytotoxic activity of the extracts against skin cells *in vitro* and potential chemopreventive effects *in vivo*. Considering the honeybush species, the flavanol-like compounds also appear to be responsible for a cytotoxic effect in skin cells, but the monomeric polyphenols, particularly the xanthones and flavanones, at a specific ratio, are likely to exhibit cytoprotective antioxidant effects. Contrary to rooibos, a different set of predictive criteria needs to be considered for honeybush species. The reduction in cell viability, involving a decrease in ATP content, is associated with mitochondrial dysfunction resulting from defects in the respiratory chain complex presumably via polyphenol–iron interactions. Future studies should focus on the effects of the herbal tea extracts on the mitochondrial membrane depolarisation, cell cycle arrest and the induction of apoptosis.

Declarations

Conflict of interest

No conflict of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HPLC chromatograms for *Camellia sinensis* aqueous (a) and methanol (b) extracts, as well as *Apalathus linearis* aqueous (c) and methanol (d) extracts.

Figure S2. HPLC chromatograms for *Cyclopia genistoides* aqueous (a) and methanol (b) extracts, as well as *Cyclopia longifolia* aqueous (c) and methanol (d) extracts.

Figure S3. HPLC chromatograms for *Cyclopia intermedia* aqueous (a) and methanol (b) extracts, as well as *Cyclopia subternata* aqueous (c) and methanol (d) extracts.