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Cancer Letters 224 (2005) 193–202
CANCER Letters
www.elsevier.com/locate/canlet

Inhibition of tumour promotion in mouse skin by extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique South African herbal teas

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Received 27 September 2004; received in revised form 30 October 2004; accepted 4 November 2004

Abstract

The modulating effect of ethanol/acetone (E/A) soluble fractions, prepared from methanolic extracts of processed and unprocessed rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) as well as green (*Camellia sinensis*) teas was established in a two-stage mouse skin carcinogenesis assay. Topical application of the tea fractions prior to the tumour promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), on ICR mouse skin initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA) suppressed skin tumorigenesis significantly ($P<0.001$) with the green tea E/A fraction exhibiting a 100% inhibition, unprocessed honeybush 90%, processed honeybush 84.2%, processed rooibos 75% and unprocessed rooibos 60%. The green tea fraction, with the highest flavanol/proanthocyanidin content, also exhibited the highest protective activity (99%) against hepatic microsomal lipid peroxidation, and completely inhibited skin tumour formation. Differences in the flavanol/proanthocyanidin and flavonol/flavone composition and/or non polyphenolic constituents are likely to be important determinants in the inhibition of tumour promotion by the herbal tea E/A fractions in mouse skin.

Keywords: Rooibos; Honeybush; TPA; Chemoprevention

1. Introduction

Chemical carcinogenesis in murine and possibly human skin is a multi step process including initiation, promotion and progression [1,2]. In mouse skin, a single topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA) effects initiation while promotion is accomplished by repeated topical applications of a promoter such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [3,4]. In contrast to initiation, which is irreversible and possibly unavoidable because of continuous exposure to chemical and physical

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doi:10.1016/j.canlet.2004.11.014

carcinogenic agents, the process of promotion is reversible [4,5]. The reversibility of tumour promotion therefore provides an opportunity to interrupt or delay the development of altered lesions resulting in tumour formation.

A variety of plant and/or other phenolic compounds exhibit chemoprotective properties by disrupting the different stages of multi step skin carcinogenesis, especially tumour promotion [5–11]. The phenolic composition of green tea has been well characterised and the major flavonoid constituents, the catechins, are known to be inhibitors of cancer initiation and promotion in mouse skin [11–13]. The major phenolic components of the unprocessed South African herbal teas are the dihydrochalcones, aspalathin and nothofagin present in rooibos (*Aspalathus linearis*) and the xanthone, mangiferin and flavanone, hesperidin in honeybush (*Cyclopia intermedia*) [14,15]. In the processed herbal teas, aspalathin remains one of the major components in rooibos. Although mangiferin and hesperidin decrease with processing, they also remain the major monomeric polyphenols in processed honeybush. The polyphenolic composition differs from that of green tea and their cancer protective properties are not known at present.

Aqueous extracts of rooibos tea significantly decreased the number of chromosomal aberrations in hamster ovary cells treated with benzo[*a*]pyrene in the presence of liver homogenates [16]. An in vivo study in male ICR mice showed a significant decrease in the number of micronucleated reticulocytes, induced by mitomycin C, by aqueous extracts of rooibos tea [16]. Apart from these studies very little is known about the modulation of the genotoxic effects of carcinogens by these herbal teas. Recent investigations showed that aqueous extracts of honeybush and rooibos exhibit antimutagenic properties against aflatoxin B_1 and 2-acetylaminofluorene induced mutagenesis in vitro [17] and ex vivo [18]. Aqueous extracts of unprocessed and processed rooibos as well as unprocessed honeybush significantly enhanced the antioxidant capacity in the liver of rats by stabilising glutathione (GSH) [19]. The activity of hepatic phase II metabolising enzymes, glutathione-*S* transferase (GST-α) and UDP-glucuronosyl transferase (UDP-GT) was also significantly enhanced in the liver by the aqueous extracts of rooibos and honeybush teas. This would imply that these herbal teas not only altered the metabolic fate of carcinogens but also the oxidative status of cells that can protect against the adverse effects of oxidative damage induced by many carcinogens.

The present study investigated the modulating effect of ethanol/acetone (E/A) soluble fractions prepared from methanol extracts of processed and unprocessed rooibos and honeybush teas on tumour promotion in mouse skin, using green tea as reference.

2. Materials and methods

2.1. Chemicals

7,12-Dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetra-decanoylphorbol-13-acetate (TPA), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), ethylene diamine tetra-acetic acid disodium salt (EDTA), gallic acid, (+)-catechin, quercetin, mangiferin, rutin, *p*-dimethylaminocinnamaldehyde (DAC) and Folin's reagent, were purchased from Sigma Chemical Co. (Cape Town, South Africa). Hesperidin and hesperetin were obtained from Aldrich Chemical Co. (Cape Town, South Africa). Orientin, iso-orientin, vitexin, iso-vitexin, luteolin, chrysoeriol and isoquercitrin were purchased from Extrasynthese (Genay, France). Aspalathin and nothofagin, isolated from unprocessed/'green' rooibos to a purity of $>95\%$, were supplied by Ms P Snijman (PROMEC Unit, MRC, South Africa). All other chemicals used were of analytical grade.

2.2. Animals

Six-week-old female ICR mice were obtained from the Animal Unit, Medical School, University of Cape Town (South Africa) and kept in the Animal Unit of the University of Stellenbosch, (Tygerberg, South Africa) for at least 1 week prior to commencement of the experiment. Mice had free access to pelleted murine chow (Epol LTD, Johannesburg, South Africa) and water while kept on a 12 h photoperiod with optimum air changes per hour and a constant room temperature of 21 °C.

2.3. Plant material

Processed (oxidised) and unprocessed ('green'/unoxidised) rooibos and honeybush teas, part of the indigenous 'fynbos' flora in certain coastal and mountainous regions of the South Western Cape Province (South Africa), were obtained from ARC Infruitec-Nietvoorbij (Agriculture Research Council), Stellenbosch, South Africa. The green tea (*Camellia sinensis* var *sinensis*), imported from China, was a gift from Vital Health Foods, Kuils River, South Africa.

2.4. Preparation of the ethanol/acetone soluble (E/A) extracts

Polyphenolic extracts of both processed and unprocessed rooibos and honeybush and Chinese green teas were prepared by extracting (three times) grounded plant material with chloroform (3% m/v) by vigorous stirring in an Erlenmeyer flask and filtering through Whatman no 3 filter paper. The residual plant material was exhaustively extracted overnight with methanol as described above, filtered, the solvent evaporated under reduced pressure at 40 °C in a Rotavapor and the residue stored in an airtight container at 4 °C in the dark. The E/A (1:1 v/v) soluble fraction of the different tea preparations was prepared by reconstituting 30 mg of the methanol extract in 1 mL absolute ethanol and acetone (1:1) mixture.

2.5. Soluble solid, total polyphenolic, flavanol/proanthocyanidin and flavonol/flavone content of the E/A soluble fractions

The solid content of each E/A soluble tea fraction was determined gravimetrically (four repetitions) after drying 1 mL aliquots at 110 °C for 12 h. The Folin-Ciocalteu method with gallic acid as standard was used to determine the total polyphenol (TP) content [20]. The flavanol/proanthocyanindin and flavonol/flavone contents were determined colorimetrically (640 nm) using DAC with (+)-catechin as standard [21] and spectrophotometrically (360 nm) using quercetin as standard [22], respectively. The results were expressed as mg catechin or mg quercetin equivalents/mg soluble solids. The possible contributions of hesperidin, mangiferin and aspalathin to the flavanol/proanthocyanidin content were also determined spectrophotometrically using reactivity with DAC as a measure.

2.6. HPLC quantification of the major flavonoids in the E/A soluble herbal tea fractions

Dried E/A fractions of rooibos and honeybush were dissolved in methanol and filtered through a Magna Nylon 13 mm 0.45 μm filter prior to HPLC analysis. The honeybush extracts were analysed for mangiferin, hesperidin and hesperetin according to the method of Joubert et al. [15]. HPLC analysis were conducted on a LaChrom2000 system comprising an L-7400 UV detector, L-7100 pump, Rheodyne 7725i injection valve and D-7000 HPLC system manager and interface using a Phenomenex Synergy Max-RP C12 80A (4 μm, 150×4.6 mm I.D.) column while quantification was done at 280 nm. The rooibos extracts were analysed for the dihydrochalcones, aspalathin and nothofagin and the flavones, orientin, iso-orientin, vitexin, iso-vitexin, luteolin, chrysoeriol and flavonols quercetin, iso-quercitrin and rutin. Rutin co-eluted with iso-quercitrin and the quantification was expressed as quercetin equivalents. A Waters LC Module 1 Plus system, 2996 diode array detector and Millennium Version 3.2 for system control and data acquisition were used. Separation was conducted on a Merck Lichrosphere 100 RP-18 (5 μm, 250×4 mm I.D.) column. Quantification was conducted at 288 nm (dihydrochalcones) and 255 nm (flavones and flavonols) according to the method of Joubert [23] with a modification to the initial gradient. A calibration curve for each flavonoid was prepared.

2.7. Thiobarbituric acid reacting substances (TBARS) determinations

The formation of TBARS, measured as malondialdehyde (MDA), was used to determine the protective effect of the E/A soluble fractions (0.01% dissolved in DMSO) against lipid peroxidation utilising a rat liver microsomal (1 mg protein/mL) preparation. Lipid peroxidation was determined using a modified method of Yen and Hsieh [24] in the presence of Fe^{2+} and absence of hydrogen peroxide. Results were expressed as nmol MDA per mg protein using

the millimolar extinction coefficient of 153 [25]. The microsomal fraction was prepared from an S-9 liver homogenate by Sepharose 2B column chromatography in Tris buffer (50 mM, pH 7.4) containing KCl (150 mM) as previously described [26]. The microsomes were stored at −80 °C until used. Protein determination was performed according to the method of Kaushal and Barnes [27] using bovine serum albumin as standard.

2.8. Effect on tumour promotion

Eighty mice were randomly divided into eight treatment groups of 10 animals each and caged separately. Prior to the study the dorsal side of the skin was treated with a depilatory cream (No Hair; Adcock Ingram, South Africa). Mice were treated with a single topical application of DMBA (200 nmol) in acetone (100 uL) as tumour initiator followed by the cancer promoter, TPA (5 nmol; 100 uL acetone), 1 week later. The protective effect of the E/A tea fractions was monitored by the topical application (100 uL) 30 min prior to TPA treatment. Positive control (DMBA/TPA) mice were treated with the carrier solvent in a similar manner (acetone/ethanol). Two experimental control groups were included consisting of DMBA/acetone and acetone/TPA treatments. The treatments were repeated twice weekly for 20 weeks and the number of skin tumours was recorded weekly. The final tumour yield and tumour volume were determined upon termination by CO_2 asphyxiation. Details of the experimental outlay are summarised in Fig. 1. The experiment was repeated once.

2.9. Statistical analyses

An Analysis of Variance (ANOVA) was performed on the data using Proc GLM in SAS. Where equal group variances were present (soluble solids, flavanol content, TBARS), the *F*-test was used to test for group differences, using the parametric Tukey-

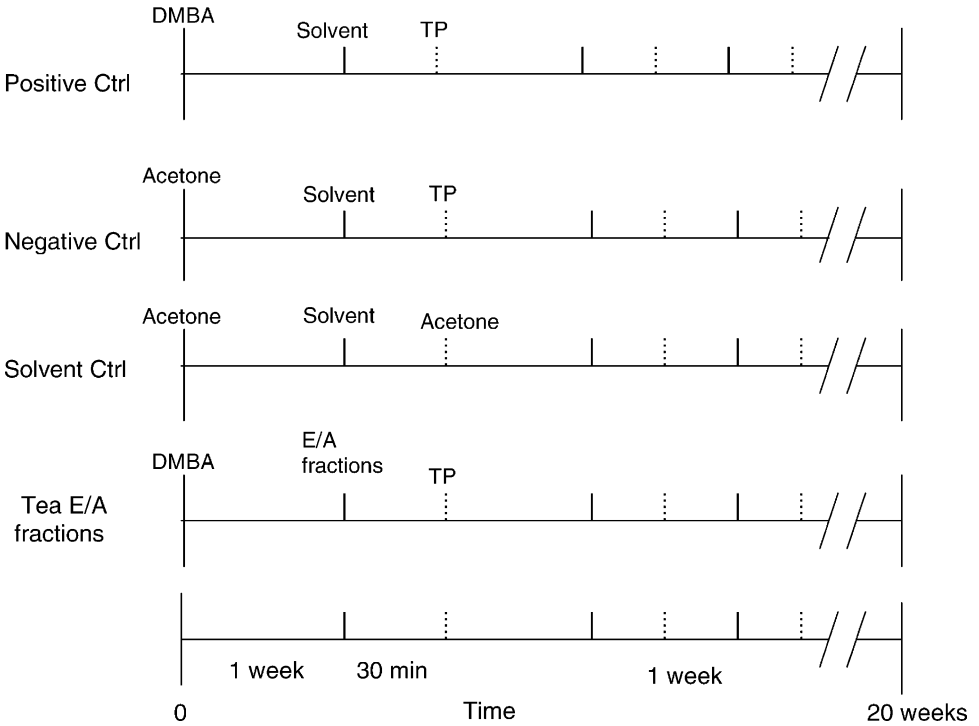

Fig. 1. Experimental protocol of the various treatment regimens used in the two-stage mouse skin cancer model. Mice were treated with a single dose of DMBA (200 nmol) or the control solvent (acetone). Modulation of cancer promotion was conducted by the application of the tea ethanol/acetone (E/A) polyphenolic fractions 30 min prior to TPA (5 nmol) twice weekly for 20 weeks.

Table 1
Soluble solids, total polyphenolic, flavanol/proanthocyanidins and flavonol/flavone contents of the ethanol/acetone tea extracts topically applied to mouse skin

Tea extracts	Soluble solids (mg/100 uL)	TP content (mg Gallic acid equivalents/mg soluble solids)	Flavanol/ proanthocyanidin content (mg Catechin equivalents/mg soluble solids)	Flavanol/proanthocyanidin content (expressed as a % of TP)	Flavonol/flavone content (mg Quercetin equivalents/mg soluble solids)	Flavonol/flavone content (expressed as a % of TP)	[$]TBARS (nmol MDA/mg protein)
Rp	2.24 ± 0.36a	0.24 ± 0.03a (24%)	0.05 ± 0.001a (5.4%)	21.90 ± 0.05a	0.037 ± 0.010a (3.7%)	15.07 ± 4.57a	1.40 ± 0.44a
Rg	2.29 ± 0.20a	0.55 ± 0.08b (55%)	0.26 ± 0.004b (26%)	46.46 ± 0.12b	0.026 ± 0.002a (2.6%)	4.68 ± 0.31b	0.37 ± 0.10b
Hp	2.16 ± 0.46a	0.13 ± 0.02(a) (13%)	0.02 ± 0.001c (2%)	13.62 ± 0.01c	0.014 ± 0.008b (1.4%)	10.52 ± 6.37a	3.50 ± 0.43c
Hg	2.07 ± 0.42a	0.31 ± 0.04a (31%)	0.28 ± 0.001b (28%)	91.04 ± 0.09d	0.015 ± 0.003 (b) (1.5%)	4.84 ± 1.12b	1.47 ± 0.31a
Gr	2.57 ± 0.41a	0.56 ± 0.05b (56%)	0.51 ± 0.002d (51%)	91.50 ± 0.16d	0.010 ± 0.002b (1%)	1.78 ± 0.4c	0.04 ± 0.01b
Ctrl	–	–	–	–	–	–	4.01 ± 0.10d

[$] The inhibitory effect on lipid peroxidation was monitored in rat liver microsomes (1 mg protein/mL) in the present of Fe^{2+}. TBARS assay conducted using 0.01% (w/v) of the E/A polyphenolic fraction dissolved in DMSO. Values are means ± SD of 17–20 determinations, except for soluble solids ($n=4$). When comparing the different groups, values in columns followed by the same letter indicate no significant difference. If letters differ then $P<0.001$. When the letters are in parenthesis then $P<0.1$. Values in parenthesis are expressed as a % of the soluble solids. Abbreviations: Rp, processed rooibos; Rg, unprocessed rooibos; Hp, processed honeybush; Hg, unprocessed honeybush and Gr, green tea. TP, total polyphenols.

test to indicate which specific groups differed significantly. Otherwise the Welch test was used where groups had unequal variances (TP content, flavonol content, tumour volume, total number of tumours). Student's *T*-test was used to test for pair wise group differences.

3. Results

No noticeable clinical signs of illness e.g. weight loss, diarrhoea, rhinitis, alopecia were detected in any of the groups as a result of the topical application of the different treatments.

3.1. Soluble solids, total polyphenolic and flavonoid content of the E/A soluble fractions

No significant ($P>0.05$) differences were noticed in the soluble solid content of the various fractions. The TP content of the E/A soluble fractions of the processed herbal teas (13–24%) were significantly ($P<0.001$) to marginally ($P<0.1$) lower when compared to their unprocessed counterparts (31–55%) as well as the green tea (56%) (Table 1). The green tea E/A fraction had a similar TP content to that of the unprocessed rooibos. The E/A fractions of the unprocessed herbal teas had a similar flavanol/proanthocyanidin content while their processed counterparts had a significantly lower ($P<0.001$) flavanol/proanthocyanidin content with processed honeybush containing the lowest amount ($P<0.001$). When expressed as a percentage of the TP content, the green tea and unprocessed honeybush tea E/A fractions contain up to 90% flavanols/proanthocyanidins, followed by unprocessed rooibos (47%), processed rooibos (22%) and processed honeybush (14%). The flavonol/flavone content of the processed and unprocessed rooibos E/A fraction was significantly ($P<0.001$) higher than that of the honeybush and green tea fractions. The unprocessed honeybush E/A fraction contained a marginally ($P<0.1$) higher flavonol/flavone content when compared with green and processed honeybush fractions. When considering the flavonol/flavone content as a percentage of the TP content the E/A fractions of the unprocessed herbal teas (~4.7%), exhibited the lowest amounts when compared to the processed counterparts

(10–15%) which were still significantly ($P<0.001$) higher than the green tea E/A fraction (1.8%).

None of the major flavonoid constituent of the E/A fractions prepared from honeybush and rooibos, aspalathin, mangiferin and hesperidin showed a positive reaction with DAC when the flavanols were determined.

3.2. Quantification of selective flavonoids of herbal E/A fractions

Aspalathin and nothofagin comprised the major flavonoids in the E/A fraction of the unprocessed rooibos (Table 2). The flavone analogues of aspalathin, orientin and iso-orientin and those derived from nothofagin, vitexin and iso-vitexin were also detected. Rutin and isoquercitrin, that co-eluted, were present while only trace amounts of quercetin, luteolin and chrysoeriol were detected. A similar pattern was noticed in the E/A fraction from the processed rooibos with the exception that aspalathin and nothofagin were reduced while their flavone analogues increased. Rutin/isoquercitrin, quercetin, luteolin and chrysoeriol could also be detected. Mangiferin and hesperidin were the major flavonoid constituents detected in the unprocessed and processed honeybush E/A fractions. As noticed for rooibos tea, the honeybush flavonoids were markedly reduced as a result of tea processing.

Based on the volume of each fraction applied to mouse skin, aspalathin was the major flavonoid in the unprocessed rooibos E/A fraction whilst iso-orientin, orientin and aspalathin represented the major flavonoids when administering the processed rooibos fraction. Mangiferin and hesperidin were the major compounds in both the unprocessed and processed honeybush fractions (Table 2).

3.3. Inhibition of microsomal lipid peroxidation

The unprocessed herbal tea fractions exhibited a higher protective effect against lipid peroxidation when compared to their processed counterparts (Table 1). Of the herbal teas unprocessed rooibos exhibited the highest protective effect (91%) while processed rooibos (65%) and unprocessed honeybush (63%) showed a similar but lower protection. The processed honeybush fraction was the least effective against the inhibition of lipid peroxidation (13%) with the green tea fraction exhibiting the highest ($P<0.05$) protective effect (99%) of all the teas fractions.

Table 2
Quantitative amount of flavonoids applied to the mice skin using processed and unprocessed herbal ethanol/acetone tea fractions

Polyphenols	E/A fractions			
	Processed		Unprocessed	
	% Soluble solids	μg/100 μL	% Soluble solids	μg/100 μL
Rooibos constituents				
Aspalathin	2.12±0.02	63.45±0.62	5.67±0.06	226.82±2.31
Nothofagin	0.24±0.01	7.36±0.03	0.81±0.01	32.47±0.15
Orientin	2.43±0.01	72.80±0.09	0.54±0.01	21.68±0.10
Iso-orientin	3.08±0.01	92.33±0.03	0.65±0.01	26.15±0.03
Vitexin	0.37±0.01	11.24±0.02	0.06±0.01	2.49±0.04
Iso-vitexin	0.94±0.04	28.29±0.12	0.27±0.01	10.76±0.04
Rutin/iso-quercitrin	0.74±0.01	22.09±0.17	0.31±0.01	12.24±0.07
Quercetin	0.13±0.01	3.83±0.25	Trace	Trace
Luteolin	0.11±0.01	3.19±0.35	Trace	Trace
Chrysoeriol	0.10±0.01	2.91±0.002	Trace	Trace
Honeybush constituents				
Mangiferin	3.74±0.01	112.19±0.52	5.61±0.08	168.42±2.37
Hesperidin	3.69±0.31	110.69±9.32	4.14±0.15	124.26±4.42
Hesperetin	Trace	Trace	0.41±0.01	12.24±0.01

The values in columns represent the mean±SD of 2–3 determinations.

Table 3
Inhibitory effect of the topical application of various tea E/A polyphenolic fractions on TPA-induced tumour promotion in mouse skin

Treatment	Mean no tumours per mouse	Mean tumour volume per mouse (mm^3)
Pos	5.3±4.7a	80.4±158.6a
Rp	0.7±1.6b	34.7±130.4a
Rg	0.9±1.6b	27±55.6a
Hp	0.2±0.5b	1.8±5.7[a]
Hg	0.3±0.9b	11.3±35.7a
Gr	0	0

Abbreviations: Rp, processed rooibos; Rg, unprocessed rooibos; Hp, processed honeybush; Hg, unprocessed honeybush and Gr, green tea. Values in columns are means±SD of 15–20 mice per group. When comparing the different groups, values in columns followed by the same letter indicates no significant difference. If letters differ then $P<0.001$.

[a] Excluded from statistical analysis as $n=1$.

3.4. Inhibition of tumour promotion.

None of the mice in the negative control groups (DMBA/solvent and solvent/TPA) developed skin tumours. The positive control mice (DMBA/TPA) developed a mean number of 5.3 skin tumours per mouse (Table 3) with a tumour incidence of 88.3% (Fig. 2). No tumours developed in the mice treated with the green tea E/A fraction. Topical application of the various herbal tea E/A fractions significantly ($P<0.001$) protected against tumour promotion by TPA (Table 3). The mean number of tumours per mouse was significantly reduced by unprocessed rooibos (1.1) followed by processed rooibos (0.7), unprocessed (0.3) and processed (0.2) honeybush fractions. Unprocessed honeybush exhibited the highest decrease (90%) in the percentage of tumour bearing mice, followed by processed honeybush (84.2%), processed rooibos (75%) and unprocessed rooibos (60%) fractions (Fig. 2). When considering the mean tumour volume/mouse a similar trend was observed, with the honeybush tea extracts showing the main decrease in tumour size followed by the rooibos tea extracts (Table 3).

The herbal tea fractions not only decreased the tumour volume and the mean number of tumours per mouse but also delayed tumour development. The first tumour in the DMBA/TPA treated mice appeared at 4 and 12 weeks when treated with the processed and unprocessed rooibos and 16 weeks when the processed and unprocessed honeybush E/A fractions (Fig. 2).

4. Discussion

The mechanisms involved in mouse skin tumour promotion have not been fully characterised but events such as hyperplasia, inflammation and production of reactive oxygen species (ROS) amongst others, have been shown to be important [12,28,29]. Pre-treatment of murine skin with natural antioxidants suppressed the oxidative stress, activity of ornithine decarboxylase (ODC), cell proliferation and ultimately inhibited skin tumour promotion [30,31]. Topical application of green and black tea polyphenols inhibited TPA-induced inflammation and protected against the onset and subsequent development of skin tumours [7,11,13,32]. Polyphenolic fractions of green tea exhibited antioxidant activity towards hydrogen peroxide and superoxide anion radicals in mouse hepatocytes and human keratinocytes [33,34]. Aspalathin, the main polyphenolic constituent of rooibos tea exhibits a similar antioxidant activity as epigallocatechin gallate (EGCG) in the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS] and microsomal lipid peroxidation assays [35]. Hesperidin and hesperetin, major honeybush

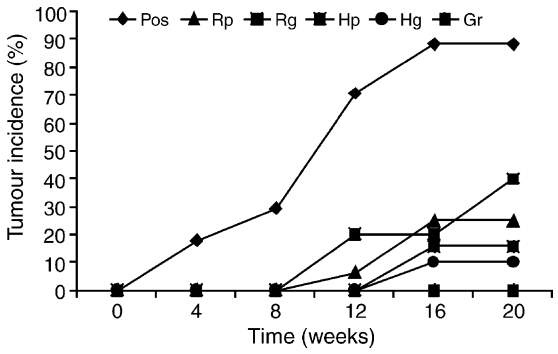

Fig. 2. Inhibitory effect of topical application of various E/A polyphenolic fractions on TPA-induced tumour promotion. The percentage mice with tumours are plotted as a function of the treatment period (weeks). The fractions include Rp, processed rooibos; Rg, unprocessed rooibos; Hp, processed honeybush; Hg, unprocessed honeybush and Gr, green tea. The number of animals per group = 15–20.

flavonoids also possess anti-inflammatory activity [36,37], with mangiferin constituting the major antioxidant of honeybush tea [38]. Thus, compounds exhibiting antioxidant and/or anti-inflammatory activities are expected to be effective anti-tumour promoting agents [39].

The present study confirms that a polyphenolic fraction of green tea, containing mainly flavanol/proanthocyanidin constituents, inhibits the formation of mouse skin tumours. Evidence is also provided for the first time, that E/A soluble fractions prepared from unprocessed and processed rooibos and honeybush methanol extracts protect against TPA-induced tumour promotion in mouse skin. A significant ($P < 0.05$) decrease in tumour incidence, a marked reduction in tumour volume and a delay in the onset of tumour development were shown in the present study. Previous studies showed aqueous extracts of the herbal teas reduce oxidative stress, as well as enhance the antioxidant capacity in rat liver after chronic exposure [18,19]. Aqueous extracts of processed rooibos tea also significantly inhibited the activity of ODC in fibroblasts and myoblasts in tissue culture which were ascribed to the potent radical scavenging activity of rooibos tea extract [40]. Data from these studies and the present study suggest that the antioxidant activity of the different tea E/A fractions are likely to be involved in the reduction of ROS induced by TPA thereby inhibiting tumour promotion.

Considering the inhibition of lipid peroxidation, the green tea fraction with the highest TP content, exhibited the best protection against lipid peroxidation and tumour promotion. Inhibition of lipid peroxidation by the herbal tea fractions are in accordance with previous data indicating a relationship between the polyphenolic content of aqueous rooibos and honeybush tea extracts and their antioxidant potency [41–45]. However, no relationship existed between the TP content and the inhibition of lipid peroxidation on the one hand and their protection against tumour promotion on the other, in the present study. It seems that differences in, and/or the relative concentrations of flavonoid subgroups constituting the TP of the herbal E/A fractions, will determine their protective potency against tumour promotion.

When considering flavonoid subgroups, the flavanol/proanthocyanidin content, expressed as a percentage of the TP content, both green tea and unprocessed honeybush tea fractions exhibited similar levels (approximately 90%). This would imply that the flavanol content as well as the type of flavanols and/or flavanol-like compounds, such as the proanthocyanidins and polymeric tea tannins could also play an important role in the inhibitory effect on tumour promotion. In this regard, the E/A fractions from green tea and unprocessed honeybush showed the strongest reaction when utilising the butanol/HCl method [46] to determine the presence of proanthocyanidins (unpublished data). The proanthocyanidins, therefore, are likely to make a major contribution towards the flavanol content of unprocessed honeybush fraction when compared with the other herbal tea E/A fractions. The proanthocyanidins from grape seed oil has been shown to inhibit photo- and chemically-induced skin carcinogenesis in mice [47,48]. Of interest is that the major flavonoids determined in the honeybush tea fractions, hesperidin and mangiferin, did not contribute to the flavanol content. A previous study also reported on the lack of hesperidin to react with DAC during the colourimetric determination of flavanols [49].

The flavonol/flavone content of both the unprocessed honeybush and rooibos fractions contained approximately 5% of the TP content. As the unprocessed honeybush fraction exhibited the highest protective effect it would imply that the flavonol/flavone play a secondary role to the flavanols/proanthocyanidins. This is further supported by the fact that the E/A fraction of green tea exhibiting the highest protective effect also contained the lowest flavonol/flavone content ($\sim$2%). A higher flavonol/flavone and lower flavanol/proanthocyanidin content exhibited intermediate effects against tumour promotion, as in the case of the processed rooibos and honeybush fractions. As described for honeybush tea, the proanthocyanidins of rooibos tea could also be involved, as tannins of unprocessed rooibos comprises of epicatechin and catechin-extending units [50].

The relative contribution of the different flavonoids subgroups and/or non-flavonoids to the anti-tumour promoting properties of the herbal teas is not known at present. Subsequent studies need to be conducted to elucidate whether the herbal tea polyphenolic fractions and/or specific flavonoids, could modulate biological events involved during TPA-induced tumour promotion in mouse skin.

Acknowledgements

The authors are grateful to the staff of Tygerberg Animal Unit for maintenance of experimental animals and Ms A Pather for technical assistance. The authors also thank Ms P Snijman for the preparation of tea methanolic extracts and Ms D de Beer and Mr CM Malherbe from ARC, Infruitec-Nietvoorbij, Stellenbosch, for HPLC analyses. This study is jointly supported by the Cancer Association of South Africa, The Medical Research Council of South Africa, The Rooibos Forum, National Research Foundation (Indigenous Knowledge Systems, GUN 2050353) and the Department of Trade and Industry (Technology and Human Resources for Industry Programme, project 2424).

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