



Rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and cancer bush (*Sutherlandia frutescens* subsp. *microphylla*) protect against tobacco-specific mutagenesis in vitro



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ARTICLE INFO

Available online 22 June 2016

Edited by D De Beer

Keywords:

Antimutagenesis
Tobacco-specific mutagens
Herbal teas
Cancer bush

ABSTRACT

Antimutagenesis studies against the tobacco-specific mutagens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-oxide, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), were conducted using hot water aqueous extracts of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*), and cancer bush (*Sutherlandia frutescens*). Aqueous extracts of both “fermented” and “unfermented” (green) rooibos and honeybush were included, while extracts of green and black teas (*Camellia sinensis*) served as benchmarks. A polyphenol-enriched methanol extract of unfermented rooibos (RgM) was included to further elucidate the possible role of rooibos polyphenols. Studies were performed in the presence of the metabolic activation against *Salmonella typhimurium* tester strain TA1535, using the standard plate incorporation and micro-suspension, pre-incubation assays. The mutagenic effects of NNK against the strain TA1535 was best demonstrated using the standard plate incorporation assay, while a higher mutagenicity was demonstrated for NNAL using the micro-suspension, pre-incubation method. Black tea and RgM exhibited the highest protection against NNK-induced mutagenesis followed by the aqueous extracts of rooibos ≥ green tea ≥ honeybush ≥ cancer bush. Black tea, green tea, RgM and unfermented rooibos were the most effective against NNAL-induced mutagenesis, followed by fermented rooibos. The two honeybush extracts exhibited similar, but the weakest protective response. When considering the amount of total polyphenols (TPP) incorporated in the plate incorporation assay, cancer bush exhibited similar protection to that of fermented and unfermented honeybush against NNK mutagenesis. The involvement of specific polyphenol-cytochrome P450 (CYP450) interactions is likely to be involved in the protection against tobacco-related mutagenesis. Polyphenol constituents of rooibos, honeybush and cancer bush could play an important role in the protection against mutagenesis induced by the major tobacco-specific carcinogens.

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1. Introduction

Nicotine is arguably responsible for more adverse health consequences when considering the estimated 3500 different compounds found in tobacco smoke (Zevin et al., 1998). It is composed of pyridine and pyrrolidine rings that are extensively metabolised in the liver into various major and minor metabolites. Oxidation accounts for 70–80% of the metabolism of nicotine resulting in the formation of cotinine via 5'-hydroxylation predominantly catalysed by cytochrome P450

2A6 (CYP2A6) (Yamazaki et al., 1999). Additionally, studies showed that CYP2A6 catalyses 2'-hydroxylation of nicotine yielding 4-(methylamino)-1-(3-pyridyl)-1-butanone, an aminoketone that, through a process of nitrosation, is converted to the potent tobacco-specific lung carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Hecht et al., 2000). The major metabolic pathway of NNK in many tissues is the reduction of the carbonyl producing N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (Hecht et al., 1997). Both NNK and NNAL are metabolically activated via similar pathways involving tissue specific CYPs, which proceed via the α -hydroxylation of either the methyl or methylene carbon. NNAL and NNK administered in the drinking water of rats produced lung tumours in 80% and 90% of the animals, respectively (Rivenson et al., 1988). The metabolic conversion of nicotine via 2'-hydroxylation and

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the subsequent formation of NNK, therefore links nicotine to the development of lung cancer. NNK exhibits a mutagenic response against strains TA100, TA1535 (Padma et al., 1989) and TA7004 following metabolic activation while it lacks mutagenicity against strains TA98 and TA1538 (Yim and Hee, 2001). In a study employing a micro-suspension assay to conduct a modified mutagenicity assay, NNAL was reported to be mutagenic against strain TA1535 after metabolic activation (Brown et al., 2001). The mutagenicity/genotoxic and carcinogenic effects of NNK are closely correlated and a similar relationship probably exists in humans (Hecht et al., 1997).

The chemopreventive properties of tea (*Camellia sinensis*) against genotoxic carcinogens, specifically tobacco-specific carcinogens, have been reported and several distinct mechanisms have been characterised (Weisburger and Chung, 2002). These include the inhibition of the metabolic activation to form the reactive DNA intermediates, increase in activity of enzymes involved in the detoxification pathways and regulation of transcription factors controlling the activity of growth factors associated with the growth of neoplastic cells. The polyphenol constituents of green and black tea are also effective inhibitors of oxidative DNA damage leading to the formation of adducts such as 8-hydrodeoxyguanine. These teas inhibit lung tumorigenesis in mice and rats induced by NNK and their major polyphenols, i.e. epigallocatechin gallate (EGCG) of green tea and theaflavins and thearubigins of black tea, as well as caffeine have been implicated as the major chemopreventive constituents (Chung, 1999). Antimutagenic properties have also been demonstrated for the South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* species) (as reviewed by Joubert et al., 2008a), but no studies have been performed on their ability to inhibit mutagenesis induced by tobacco-specific mutagens. In addition, *Sutherlandia frutescens*, commonly known as the cancer bush, is another indigenous South African plant of interest. Leaf decoctions or infusions are used to treat a number of different diseases, including cancer (Van Wyk and Albrecht, 2008). The abovementioned plants represent unique chemical diversity, differing from *C. sinensis* tea in that the major types of phenolic constituents of rooibos are, amongst others, dihydrochalcones and flavones (Joubert et al., 2012), those of honeybush, xanthenes and flavanones (De Beer and Joubert, 2010), while cancer bush contains sutherlandins (flavonols) and sutherlandiosides (cycloartanes) (Albrecht et al., 2012).

The present study compared the protective properties of aqueous extracts of rooibos, honeybush and cancer bush against the mutagenic properties of NNK and NNAL against TA1535, utilising different variations of the *Salmonella typhimurium* mutagenicity assay. A polyphenol-enriched methanol extract of unfermented rooibos was also included to further evaluate the role of rooibos polyphenols, while aqueous extracts of green and black teas were used as benchmarks.

2. Materials and methods

2.1. Chemicals

Nicotine [54-11-5] and trifluoroacetic acid were purchased from Merck Chemicals (Pty) Ltd. (Cape Town, South Africa) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [64091-91-4] and N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) [76014-81-8] from Toronto Research Chemicals Inc. (Ontario, Canada). Nutrient Agar and Nutrient Broth No. 2 were purchased from Difco Laboratories (Detroit, USA) and Oxoid (Hampshire, UK), respectively. The *S. typhimurium* strain TA1535 was kindly donated by Dr. B.N. Ames (Berkeley, USA). Dimethylsulfoxide and acetonitrile 'Far UV' for gradient analysis were obtained from BDH Laboratories. Calibration standards for HPLC analysis of cancer bush were supplied by PlantaMedChem (Giessen, Germany; rutin), Extrasynthese (Genay, France; kaempferol-3-rutinoside), Phytolab (Vestenbergsgreuth, Germany; sutherlandioside B and Sutherlandioside D).

2.2. Plant material and extracts

Super grade fermented and unfermented rooibos (*A. linearis*) were supplied by Rooibos Ltd. (Clanwilliam, South Africa). Fermented and unfermented honeybush (*Cyclopia intermedia*) were processed according to a standard procedure (Le Roux et al., 2008), using the same batch of fresh plant material. Prior to extraction the coarsely cut, dried honeybush plant material was sieved to remove the coarse stems. Black tea (*C. sinensis*), a blend of locally produced African and Sri Lankan teas (<http://www.five-roses.com/about-us/default.htm>), was obtained from a commercial retail outlet (Cape Town, South Africa). Vital Health Foods (Kuilsriver, South Africa) supplied green tea (*C. sinensis*), imported from China, while Parceval Pharmaceuticals (Wellington, South Africa) supplied cancer bush, i.e. plant material of a wild type *S. frutescens* subsp. *microphylla*.

All aqueous extracts and the methanol extract of unfermented rooibos were prepared as described by Sissing et al. (2011). Briefly, the aqueous extracts were prepared by steeping the dried plant material in freshly-boiled water (10 g/1000 mL) for 30 min, followed by filtration and freeze-drying. For preparation of the methanol extract of unfermented rooibos (RgM) the dried plant material was defatted with chloroform whereafter it was extracted exhaustively with methanol. The methanol was evaporated under vacuum at 40 °C to obtain a dry residue. The latter residue and the freeze-dried extracts were stored desiccated at 4 °C in the dark.

2.3. Chemical analysis of plant extracts

The total polyphenol (TPP) content of the extracts was determined by using the scaled-down Folin-Ciocalteu method with gallic acid as the standard (Singleton and Rossi, 1965). The TPP of the aqueous extracts of rooibos and honeybush, as well as the major monomeric polyphenol constituents of rooibos and honeybush extracts were reported previously (Sissing et al., 2011). For comparative purposes in the mutagenesis assay, the TPP content was used to "standardise" the different types of extracts as they contain diverse phenolic compounds. The presence of sutherlandins (flavonol glycosides) and sutherlandiosides (cycloartane glycosides) was confirmed by LC-MS according to the method of Albrecht et al. (2012) (Supplementary data in Appendix: Table A.1, Figs. A.1 and A.2).

The major sutherlandins and sutherlandiosides were then quantified, using an HPLC-DAD method, run on an Agilent 1200 system consisting of a quaternary pump with incorporated in-line degasser, autosampler, column thermostat, and diode-array detector (DAD), controlled by OpenLabs CDS Chemstation software (Agilent Technologies, Waldbronn, Germany). Separation was achieved at 1 mL/min and 35 °C on an Agilent Zorbax SB (100 × 4.6 mm; 1.8 µm particle size) column (Agilent), protected by a guard cartridge of the same stationary phase and an Aquity UPLC guard filter (Waters, Milford, MA, USA). The gradient solvent system consisted of acetonitrile (solvent A) and 0.02% trifluoroacetic acid (solvent B). A multilinear gradient was performed as follows: 5–12% A (0–5 min), 12–24% A (5–15 min), 24–65% A (15–46 min), 65–100% B (46–51 min), reconditioning of 100–5% B (51–55 min) and 5% B isocratic (56–66 min).

Calibration curves were set up for all standards to test the linearity of the ultraviolet (UV)-DAD response. UV spectra were recorded between 200 and 700 nm with selective wavelength monitoring at 225 and 260 nm for quantification of sutherlandiosides and sutherlandins, respectively. Where an authentic standard was not available, results were expressed in terms of the equivalents of the calibration standard. Rutin and kaempferol-3-rutinoside were selected as standards as they are flavonol glycosides with the same aglycones as sutherlandins A and B, and sutherlandins C and D, respectively (Avula et al., 2010; Fu et al., 2010). Sutherlandiosides A and C were quantified in terms of sutherlandioside B and D, respectively.

2.4. Mutagenicity assays

The mutagenicity of nicotine, NNK and NNAL was assessed in the *S. typhimurium* mutagenicity assay using the standard plate incorporation technique against tester strain TA1535 (Maron and Ames, 1983). Mutagenicity of the test compounds was also conducted using the micro-suspension pre-incubation assay according to a modified *S. typhimurium* mutagenicity assay. Plates (five replicates for each sample) were incubated at 37 °C for 48 h where after the histidine revertant colonies were scored using a Quebec Colony Counter (American Optical Corp., Buffalo, New York).

2.4.1. Standard plate incorporation assay

Varying concentrations (1, 2 and 4 mM) of the mutagens were tested against the TA1535 strain. Each assay required 0.1 mL of each mutagen concentration, 0.1 mL of an overnight bacteria culture and 0.5 mL S-9 mix (containing 5% S-9 liver homogenate) added in the top agar layer (2 mL). The mixture was vortexed, poured and dispersed on minimal glucose agar plates incubated at 37 °C for 48 h and the number of histidine revertant colonies recorded. The liver S-9 homogenate (0.77 nmol cytochrome P450/mg protein) was prepared by inducing male Fisher rats (ca. 200 g each) with Aroclor-1245 as previously described (Maron and Ames, 1983). The control plates contained the bacteria, S-9 mix and DMSO (solvent control) or water (for NNAL assay) to monitor the spontaneous revertant counts. NNAL was dissolved in water to prevent the inhibitory effects of DMSO on its bioactivation.

2.4.2. Pre-incubation assay

The overnight bacterial culture in nutrient broth was centrifuged aseptically at 10,000 ×g for 10 min to harvest the bacteria, followed by their re-suspension in phosphate buffer (0.02 M, pH 7.4) to yield a five-fold cell concentration. The micro-suspension, consisting of 0.1 mL bacteria strain TA1535, 0.1 mL S-9 mix (final concentration of 5 or 10%), and 0.1 mL mutagen (NNK or NNAL), was incubated for 90 min at 37 °C. The top agar was added to the micro-suspension mixture and dispersed onto the minimal glucose agar plates. Control treatments contained the solvent control (DMSO or water), the tester strain and S-9 mix. The plates were incubated as described above.

2.5. Antimutagenicity assay

Stock solutions of the different freeze-dried extracts, except that of cancer bush, were prepared in distilled water at concentrations of 0.5, 1 and 2% (m/v), filtered sterilised (0.45 µm) and incorporated (0.1 mL) in the mutagenicity assay. RgM was dissolved in DMSO, while the cancer bush aqueous extract was prepared at concentrations of 5 and 10% (m/v) using distilled water in order to obtain TPP levels per plate comparable to that of the rooibos and honeybush extracts. All the extracts were tested for cytotoxic effects against the TA1535 strain by monitoring the effect on the spontaneous revertant count and intact background lawn growth, using the standard plate incorporation technique as described above. The potential antimutagenic effects of the extracts were tested against the mutagenic effect of NNK using TA1535 in the standard plate incorporation (all three concentrations) and the pre-incubation (0.5 and 1%) techniques. Control plates contained only the solvent (H₂O or DMSO), TA1535 and the S-9 mix (5%). Except for cancer bush, the antimutagenic properties of the plant extracts (0.5%) were conducted in the pre-incubation assay using NNAL at a concentration of 2 mM.

2.6. Statistical analysis

Data were analysed by two-way ANOVA and statistical differences between means determined by using the Tukey's Studentised range test. Data displaying unequal variances were tested using the non-

parametric Tukey-type test. $P < 0.05$ was deemed as significant. Correlations were conducted using the Pearson correlation coefficient.

3. Results

3.1. Chemical characterisation of extracts

The content values of the major phenolic compounds in rooibos and honeybush extracts have been previously provided by Sissing et al. (2011). Briefly, the major flavonoids in rooibos were aspalathin, nothofagin, iso-orientin and orientin. The unfermented rooibos and RgM extracts also contained substantial levels of quercetin glycosides. Methanol extraction was effective in increasing the total polyphenol content of the extract, as well as its flavone aglycone content, specifically that of luteolin. Even with this increase luteolin was present at only 0.12% considering that its glucosides, orientin and iso-orientin were present at > 1%. The xanthones, mangiferin and isomangiferin were the major phenolic compounds in honeybush extract. The low hesperidin content is attributed to removal of most of the stems before extraction. De Beer et al. (2012), analysing extracts of *Cyclopia subternata*, showed that hesperidin content is higher in the stems than leaves. Notably is the lower phenolic content of rooibos and honeybush extracts prepared from fermented plant material. "Fermentation" reduced the dihydrochalcone and xanthone content of rooibos and honeybush, respectively (Joubert et al., 2008a). The major compound in cancer bush extract was the cycloartane glycoside, sutherlandioside B (2.99%). The sutherlandiosides and sutherlandins comprised 3.81% and 3.21% of the extract, respectively (Table 1). The total polyphenol content of the extracts differed substantially with the RgM and the cancer bush extract having the highest and lowest TPP content, respectively (Table 2).

3.2. Mutagenicity of tobacco carcinogens

Nicotine was not mutagenic against the bacterial strain TA1535 in the standard plate incorporation assay or pre-incubation assay at any of the concentrations tested (Table 3). NNK exhibited a mutagenic response in both assays ($P < 0.05$), yielding a much higher and a dose response effect in the plate incorporation assay. In the pre-incubation assay there was a significant increase in the mutagenic response when the S-9 concentration was increased from 5 to 10%. NNAL only exhibited a weak mutagenic response at the highest concentration of 4 mM when using the plate incorporation assay. In the pre-incubation assay NNAL exhibited a weak mutagenic response at all the concentrations although no dose response effects were noticed. Increasing the level of S-9 concentration also did not alter the mutagenic response.

Table 1

Major phytochemical constituents of cancer bush (*Sutherlandia frutescens*) aqueous extract.

Compound	Content (mg/100 g extract)
Sutherlandin A ^a	706
Sutherlandin B ^a	913
Sutherlandin C ^b	902
Sutherlandin D ^b	691
Sutherlandioside A ^c	49
Sutherlandioside B	2985
Sutherlandioside C ^c	166
Sutherlandioside D	611

^a Sutherlandins A and B, containing a quercetin aglycone, were quantified in terms of quercetin-3-rutinoside (rutin).

^b Sutherlandins C and D, containing a kaempferol aglycone, were quantified in terms of kaempferol-3-rutinoside.

^c Sutherlandiosides A and C were quantified in terms of sutherlandiosides B and D equivalents, respectively.

Table 2

Inhibition of aqueous herbal plant and tea extracts against NNK and NNAL utilising the plate incorporation and pre-incubation assay techniques with *S. typhimurium* strain TA1535. Values in parentheses are the mean total polyphenol content (mg gallic acid equivalents) of each extract per plate.

Herbal extracts	TPP content (mg gallic equiv/100 mg extract)	Plate incorporation assay			Pre-incubation assay		
		NNK (1 mM)			NNK (1 mM)		NNAL (2 mM)
		% inhibition			% inhibition		% inhibition
		0.5%	1%	2%	0.5%	1%	0.5%
Rooibos (f)	31.75 ± 1.97a	50.3 ± 4.8a ^a (0.16)	84.3 ± 3.3a ^b (0.32)	99.3 ± 1.0a ^c (0.64)	80.4 ± 7.4a ^a (0.16)	86.7 ± 2.3a ^a (0.32)	73.02 ± 11.4a ^b (0.16)
Rooibos (uf)	33.26 ± 3.03a	63.2 ± 6.2b ^a (0.17)	85.8 ± 4.3a ^b (0.33)	99.7 ± 0.6a ^c (0.67)	39.8 ± 8.3b ^a (0.17)	72.6 ± 14.2a ^b (0.33)	85.6 ± 8.3b ^b (0.17)
Honeybush (f)	15.67 ± 2.68b	22.2 ± 3.9c ^a (0.08)	47.9 ± 5.0b ^b (0.16)	70.7 ± 0.6b ^c (0.31)	29.4 ± 12.8b ^a (0.08)	41.6 ± 9.7b ^{ab} (0.16)	55.2 ± 16.2a ^b (0.08)
Honeybush (uf)	29.40 ± 3.45 ac	35.8 ± 5.4d ^a (0.15)	57.0 ± 10.2b ^b (0.29)	97.4 ± 2.3a ^c (0.59)	33.7 ± 13.7b ^a (0.15)	55.4 ± 8.3b ^b (0.15)	53.1 ± 6.4a ^b (0.15)
Green	23.27 ± 3.39c	39.1 ± 7.6d ^a (0.12)	56.0 ± 7.0b ^b (0.23)	100.5 ± 1.9a ^c (0.47)	31.1 ± 7.7b ^a (0.12)	94.3 ± 5.9a ^b (0.12)	82.2 ± 14.1b ^b (0.12)
Black	26.65 ± 1.95ca	88.7 ± 4.3e ^a (0.13)	98.1 ± 2.3c ^b (0.27)	100.9 ± 0.2a ^b (0.53)	84.2 ± 12.3a ^a (0.13)	93.5 ± 4.4a ^{ab} (0.13)	105.2 ± 11.6b ^b (0.13)
RgM (uf)	45.62 ± 17d	88.1 ± 6.2e ^a (0.23)	98.6 ± 1.1c ^b (0.46)	101.3 ± 0.8a ^b (0.91)	63.8 ± 8.6c ^a (0.23)	90.6 ± 7.0a ^b (0.23)	81.1 ± 11.2b ^b (0.23)
Cancer bush	3.77 ± 0.19d		% inhibition 5% 35.6 ± 4.7 ^a (0.19)	10% 61.3 ± 7.1 ^b (0.38)	nd		nd

Values (% inhibition) are means ± STD of 5 replications of at least two experiments. Values in the columns followed by the same letter do not differ significantly, if letters differ then $P < 0.05$. Values in a row followed by the same letter (superscript) do not differ significantly, if letters differ then $P < 0.05$ within each assay. Fermented (f); unfermented (uf); RgM: methanol extract of unfermented rooibos. Not determined (nd).

3.3. Antimutagenicity of plant and tea extracts

Dose response effects and inter extract comparisons: no cytotoxic effects were noticed at the concentrations of the herbal extracts tested when considering the background lawn growth and spontaneous revertant count. Dose response inhibitory effects were noticed against NNK-induced mutagenesis using both the plate incorporation and pre-incubation assays (Table 2). In the plate incorporation assay, unfermented rooibos exhibited a significantly higher protection at 0.5% compared to its fermented counterpart, while no difference was observed at higher concentrations, implying a threshold effect. In contrast, in the pre-incubation assay fermented rooibos exhibited a higher protective effect. Fermented honeybush showed a weaker protection than its unfermented counterpart in the plate incorporation assay, while no significant difference was observed in the pre-incubation assay. Green tea exhibited a weaker protection than black tea in both assays. A far weaker protection was noticed for the cancer bush extract at 5 to 10% with an approximate 60% inhibition obtained at the higher concentration.

At 0.5% concentration, black tea and RgM displayed the highest percentage inhibition ($P < 0.05$) against NNK using the plate incorporation assay. Both rooibos extracts showed a higher ($P < 0.05$) protection than unfermented honeybush while green tea and fermented honeybush exhibited the weakest protection. A similar order of protection was noticed at the 1% concentration level while at the 2% level the tea and

herbal tea extracts except fermented honeybush completely inhibited the mutagenic response of NNK. In the pre-incubation assay black tea and fermented rooibos exhibited the highest, but similar protective response at the 0.5% concentration, followed by the RgM extract. Unfermented rooibos, both honeybush extracts and green tea exhibited a similar but weaker protection. However, at the 1% level green tea exhibited a similar protection to black tea and rooibos (fermented and RgM) followed by unfermented rooibos, while the honeybush extracts exhibited a weaker protection.

Differences existed between the various extracts when considering the amount of TPP included in the plate incorporation assay at the levels tested. Rooibos TPP tended to be less active when compared to black tea, but they were similar in activity to that of green tea. Comparable antimutagenic properties of fermented (1%) and unfermented (0.5%) honeybush are noticed when considering the TPP levels incorporated per plate. In this regard the antimutagenic activity of the cancer bush extract (10%) against NNK-induced mutagenesis was slightly lower when compared to that of fermented (1%) and unfermented (0.5%) honeybush. However, rooibos, green tea and black tea exhibited a far higher protection at a 0.5% level at the same TPP level. The TPP level weakly correlated at the 0.5% ($r = 0.58$, $P < 0.006$) and 1% levels ($r = 0.51$, $P < 0.02$) with the inhibition of NNK mutagenesis. A similar correlation ($r = 0.50$, $P < 0.02$) existed for the pre-incubation assay for NNK.

Black tea, unfermented rooibos, RgM and green tea displayed the highest protection at a concentration level of 0.5% against NNAL-

Table 3

Mutagenic effects of nicotine, NNK and NNAL using the plate incorporation and the pre-incubation assays.

Assay	Plate incorporation			Pre-incubation (5% S9)			Pre-incubation (10% S9)		
	Nicotine	NNK	NNAL	Nicotine	NNK	NNAL	Nicotine	NNK	NNAL
Concentrations									
1 mM	8.4 ± 1.8a ^a	316.4 ± 17.7a ^b	22.4 ± 7a ^c	16.3 ± 1.3a ^a	172.6 ± 34.6b ^b	36.3 ± 15.3a ^c	15.6 ± 3.2a ^a	234.4 ± 24.9b ^b	37.8 ± 8.6a ^c
2 mM	14 ± 4.1a ^a	426.8 ± 44.8b ^b	23.2 ± 7.6a ^c	14.2 ± 3.6a ^a	185 ± 34.6b ^b	39.8 ± 9.1a ^c	14 ± 4.1a ^a	214.8 ± 25.9b ^b	41.0 ± 7.4a ^c
4 mM	10 ± 2.5a ^a	567.6 ± 51.7c ^b	47.2 ± 15.2b ^c	14.2 ± 3.1a ^a	171 ± 24.3b ^b	35.8 ± 9.7a ^c	15.4 ± 4.03a ^a	216.5 ± 17.9b ^b	42.8 ± 1.0a ^c
Spontaneous revertants	9.8 ± 3.03a ^a			15.2 ± 1.8a ^a			16. ± 0.71a ^a		

Means ± STD in the columns followed by the same letter does not differ significantly. Means in a row denoted different letters (superscript) are significantly different ($P < 0.05$). The mutagenic response of the different mutagens used in each assay was compared separately. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); N-oxide, 4 (methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

induced mutagenesis. By comparison, the honeybush extracts exhibited a far lower protective effect. Fermented and unfermented honeybush were equally effective although the TPP of the fermented extract was almost 2 fold lower. On the other hand, fermented rooibos exhibited a weaker response against NNAL-induced mutagenesis when compared to unfermented rooibos at a similar TPP level. Of interest is that RgM also exhibited a similar protection than unfermented rooibos despite the higher TPP level per plate. No correlation was noticed between the TPP level of the extracts and inhibition of NNAL mutagenesis.

Inter assay assessments and antimutagenic comparisons showed that the plate incorporation method proved to be more sensitive in determining the protection of unfermented rooibos and RgM extracts against NNK-induced mutagenesis at the 0.5% level (Fig. 1). In contrast, a significant ($P < 0.05$) higher protection was observed for fermented rooibos when utilising the pre-incubation assay. Both honeybush and black tea extracts displayed similar protection in both the plate incorporation and pre-incubation method assay at 0.5% and 1% (Fig. 1; Table 2). Green tea was more effective against NNK-induced mutagenesis in the pre-incubation assay at the 1% concentration level. However, a similar response was noticed at the 0.5% concentration, irrespective of assay procedure. Unfermented rooibos, RgM, unfermented honeybush, fermented honeybush and green tea extracts offered a higher protection against NNAL in the pre-incubation assay when compared to NNK at the 0.5% concentration.

4. Discussion

The well-documented antimutagenic properties of *C. sinensis* have been attributed to their polyphenolic content (Ioanidde and Yoxall, 2003), in particular EGCG and the oxidised flavanol products, namely theaflavins and thearubigins. Green tea reduced NNK-induced mutagenesis by 10 to 50% in mouse liver, lung and oesophagus (Von Pressentin et al., 2001). Both green and black tea inhibited NNK-induced lung tumours in mice and rats (Chung, 1999). These studies suggest that the disruption of the metabolism of tobacco-specific N-nitrosamines yielding the ultimate carcinogenic metabolites prevents their carcinogenic properties in experimental animals. Extracts of rooibos and honeybush (*C. intermedia*) were also reported to inhibit the development of oesophageal carcinogenesis in rats, utilising the site specific carcinogen methyl benzyl nitrosamine (Sissing et al., 2011).

Nicotine lacks mutagenicity against the bacterial strain TA1535 in the standard plate incorporation assay or pre-incubation assay, which is in agreement with previous studies (Doolittle et al., 1995). The mutagenicity of NNK is well documented utilising the plate incorporation assay (Yim and Hee, 2001), while that of NNAL could only be demonstrated using a modified pre-incubation version of the *Salmonella* mutagenicity assay (Brown et al., 2001). The present study showed a weak mutagenic response for NNAL, lacking a clear dose mutagenic response in both assays. The moderate to weak mutagenic responses of NNK and NNAL is likely due to the S-9 preparations prepared from Aroclor 1254-induced rats as the latter was shown not to induce the CYP2A6 isoform responsible for the activation of these mutagens in the liver of rats (Easterbrook et al., 2001).

The antimutagenic properties of rooibos and honeybush have been reviewed (Joubert et al., 2008a). Extracts of fermented and unfermented rooibos and honeybush protected against 2-acetylaminofluorene (2-AAF) and aflatoxin B₁ (AFB₁)-induced mutagenesis using the tester strains TA98 and TA100 in the presence of metabolic activation. The honeybush polyphenols, mangiferin, eriocitrin and narirutin, exhibit moderate antimutagenicity against aflatoxin B₁ whereas hesperetin and eriodictiol exhibited similar protective properties than the green tea flavanol, EGCG (Van der Merwe et al., 2006). The rooibos flavonol glycoside, rutin, suppresses the enhanced mutagenic effects of β -carotene on NNK in human alveolar basal epithelial cells by decreasing the intracellular DNA strand breaks (Yeh et al., 2006). The current study provides the first evidence that extracts of these herbal teas may protect against mutagenesis of the tobacco specific mutagens.

Differences were noticed when considering the protective effects of the herbal tea extracts against the mutagenic effects of NNK and NNAL. Apart from fermented rooibos all the extracts were more effective against NNAL than NNK when utilising the pre-incubation test. It is known that differences exist in the protection against different mutagens as rooibos was found to be more effective against the mutagenicity of AFB₁ than 2-AAF (Joubert et al., 2008a). However, the weak mutagenic response of NNAL compared to NNK could explain the apparent more effective protection against NNAL-induced mutagenesis by the different extracts. In general, rooibos exhibits a higher antimutagenic effect than honeybush, which is ascribed to differences in their polyphenol constituents, considering that, although both rooibos extracts had a similar TPP content than unfermented honeybush, it exhibited a significant higher protection. Fermented honeybush contained far less TPP and hence exhibited a weaker protection. Total polyphenol enrichment of unfermented rooibos further implies the role of these type of compounds in the protection against NNK as RgM was more effective in both the plate incorporation and pre-incubation assays.

Black tea, with a similar TPP content than green tea, offered more protection against mutagenicity, which is in agreement with a previous investigation indicating black tea to be more effective against 2-AAF and AFB₁-induced mutagenesis (Joubert et al., 2008a). Black tea contains flavanol oxidation products, i.e. theaflavins and the more complex polymeric constituents, thearubigins. When compared to both rooibos and unfermented honeybush, black tea was more effective in protecting against the tobacco-specific mutagens. This also became apparent when comparing the antimutagenic properties of black tea and RgM extracts with the latter, containing a significant higher level of TPP, exhibiting a similar protective effect. As reported previously, "fermentation" (oxidation) of the herbal teas resulted in a substantial reduction in the total polyphenol and individual polyphenol content, as well as their antimutagenic properties (Joubert et al., 2008b).

The type of mutagen and mutagenicity assay used is of importance. Unfermented rooibos exhibited a weaker protection against NNK, while it showed a higher protection against NNAL in the pre-incubation assay. In the plate incorporation assay, unfermented rooibos exhibited a weaker protection against NNK-induced mutagenesis when compared to the pre-incubation assay. Green tea, however, exhibited a weak response against NNK in both assays, while exhibiting a higher

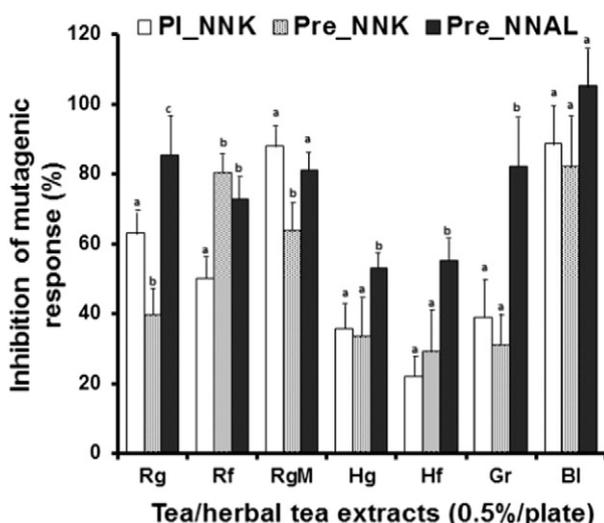


Fig. 1. Comparative antimutagenic properties of the different plant extracts (0.5% m/v per plate) against NNK and NNAL. Values are means of 5 replications. Abbreviations: PI_NNK, antimutagenicity against NNK using the plate incorporation assay; pre_NNK and pre_NNAL, antimutagenicity against NNK and NNAL using the pre-incubation assay; Rg, unfermented rooibos; Rf, fermented rooibos; RgM, methanol extract of unfermented rooibos; Hg, unfermented honeybush; Hf, fermented honeybush; Gr, green tea; BI, black tea.

protection against NNAL in the pre-incubation assay. These variations could also explain the relative weak correlation between the TPP content of the extracts and protection against mutagenesis.

Aqueous extracts of cancer bush displayed a weak antimutagenic response against NNK-induced mutagenicity compared to the herbal, green and black teas likely due to its low TPP levels. However, when considering the amount of TPP per plate it exhibited only a slightly lower protection than fermented and unfermented honeybush extracts (Table 3). Only one study reported on the antimutagenicity of cancer bush, showing that a dichloromethane extract inhibited the mutagenicity of 2-aminoanthracene using TA98 and TA100 (Reid et al., 2006). The sutherlandins, characterised as kaempferol and quercetin flavonol glycosides (Avula et al., 2010; Fu et al., 2010), could be responsible for the reported antimutagenic properties (Reid et al., 2006). However, the antimutagenic properties of sutherlandins and sutherlandiosides have not yet been investigated. A recent study reported on the binding of cancer bush extracts to the adrenal steroidogenic cytochrome P450 enzymes, thus interfering with the metabolism of pregnenolone and progesterone (Prevo et al., 2008). This suggests that constituents present in cancer bush extract could also interfere with metabolic activation of NNK and NNAL.

Herbal tea polyphenols have been reported to reduce the mutagenic potency by interfering with carcinogen metabolism (Joubert et al., 2008a). Variation in the extent of protection against NNK- and NNAL-induced mutagenesis could be ascribed to specific mutagen/polyphenol/CYP450 interactions that will vary depending on specific hydrophobic/hydrophilic environments. As polyphenols seem to interfere with the metabolic activation of mutagens (Wang et al., 1988), the interaction between the mutagens, the polyphenols and CYP450 would eventually determine the outcome of the mutagenic response. The mechanisms involved in the antimutagenic properties of the herbal extracts may therefore be attributed to the direct interaction between the polyphenols and the CYPs involved in the formation of the reactive mutagenic metabolites of NNK and NNAL. Although very little data exist regarding the interaction between tea polyphenols and CYPs, studies demonstrate that green tea polyphenols, including EGCG, inhibit the activity of both human and rat hepatic CYP1A1, with the polyphenols exerting their effects by binding at the active site of the CYP isoform (Wang et al., 1988; Muto et al., 2001). Studies also showed that EGCG inhibited the human CYP2A6, a principle cytochrome involved in NNK metabolism (Hecht et al., 2000; Muto et al., 2001).

Possible mechanisms involved in the protection against mutagenesis by polyphenols range from their ability to scavenge electrophiles or free radicals, interfering with the P450 activation system required for the metabolic activation of mutagens and carcinogens thereby altering their metabolic pathway, or catalysing the decomposition of the reactive intermediates (Edenharder and Tang, 1997). Extracts of green tea, rooibos and different *Cyclopia* spp. were demonstrated to interfere with the type II substrate binding in Aroclor 1245-induced rat liver microsomes, indicating an interaction of the constituents with CYP450 (Van der Merwe, 2004). Flavone aglycones, luteolin and chrysoeriol of rooibos, and flavanone aglycones, eriodictiol and hesperetin of honeybush, exhibited the highest interference with the type II substrate binding. Aspalathin and mangiferin also exhibited inhibitory effects on the type II substrate binding, although to a lesser extent. The antimutagenic properties of rooibos and honeybush could therefore, similar to green tea flavanols, be ascribed to the disruption of the metabolic activation of NNK and NNAL via competitive binding to CYP450. Both mangiferin and aspalathin have been shown to be bioavailable (Han et al., 2010; Breiter et al., 2011), suggesting their possible role in the protection against the in vivo mutagenesis of the main tobacco carcinogens.

Although the inhibition of mutagen activation seems to be a key mechanism in the current study, direct extrapolation of the antimutagenic properties of the herbal plants to their anti-cancer properties in vivo should take cognizance of other biological properties of the

respective major polyphenols. These include bioavailability and bio-transformation governing their excretion, the modulation of phase I and phase II enzymes of carcinogen metabolism, and the alteration of the oxidative status of the target cells. Despite these different parameters that prevail in vivo, studies indicated that both rooibos and honeybush protect against carcinogenesis in the liver, skin and oesophagus experimental carcinogenesis models (Marnewick et al., 2005, 2009; Sissing et al., 2011), suggesting a key role of their respective polyphenolic constituents that, apart from their anti-mutagenic properties against tobacco-specific carcinogens, should be further explored.

Conflict of interest

No conflict of interest to disclose.

Acknowledgements

The authors would like to thank the Cancer Association of South Africa and the South African Rooibos Tea Council for financially supporting this research, and Marietjie Stander of the Central Analytical Laboratory of Stellenbosch University for LC–MS analysis of cancer bush extract.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.sajb.2016.06.004>.

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