Influence of Processing Stages on Antimutagenic and Antioxidant Potentials of Rooibos Tea

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The antimutagenic and antioxidant potentials of rooibos (*Aspalathus linearis*) tea samples, collected from each of its major processing stages, were evaluated according to the *Salmonella typhimurium* mutagenicity test and the hydrogen donating ability and superoxide anion radical scavenging assays, respectively. Ten random samples were collected before and after fermentation, as well as after sun-drying, sieving, and steam pasteurization. Results indicated that the fermented tea had a significantly ($P < 0.05$) lower antimutagenic and antioxidant potential than the unfermented tea. Of the different processing stages, the most significant reduction in the antimutagenic and antioxidant property of the tea was found during the "fermentation" step. Sun-drying, sieving, and steam pasteurization also reduced the antimutagenic potential of the tea, although not to the same extent as the first processing step. The hydrogen donating ability was significantly increased after steam pasteurization in comparison to those of fermented and sun-dried tea. Pasteurization did not affect superoxide anion radical scavenging in comparison to fermented tea. Differences seem to exist in the antimutagenicity and antioxidant potencies of the tea sampled at the various stages during processing. A possible role of tea polyphenols in the antimutagenic and antioxdant activities of the tea is suggested as processing caused a significant reduction in the total polyphenolic content.

Keywords: *Ames test; antioxidants; polyphenols; superoxide anion radical; DPPH radical; Aspalathus linearis*

INTRODUCTION

Green and fermented or black teas are consumed worldwide and have been reported to possess various biological, pharmacological, and anticancer effects (*1*, *2*). Although many of these effects need to be scientifically verified, a protective effect of tea extracts against chronic diseases such as cancer and atherosclerosis has been proposed. The indigenous plant, *Aspalathus linearis*, that forms part of the fynbos vegetation in the southwestern and southern regions of the Western Cape of Africa is cultivated for production of rooibos, a herbal tea that is commonly consumed in South Africa and also attracts interest from consumers elsewhere. Recent findings using rooibos tea extracts indicated that it reduces the number of chromosome abberations in Chinese hamster ovary (CHO) cells treated with benzo[*a*]pyrene (*3*). Komatsu et al. (*4*) showed that rooibos extract suppressed oncogenic transformation of mouse C3H10T1/2 cells induced by X-rays. Extracts of rooibos tea were shown to exhibit antispasmodic activity in in vitro assays (*5*).

The flavanol compounds epigallo- and epicatechins appear to be the components responsible for the biological effects described for green and black teas (*1*). The polyphenolic constituents of rooibos (*6*), of which aspalathin represents one of the major compounds in unfermented rooibos (Joubert, 1996), differ from those of green and black teas (*8*). Aspalathin has been shown to exhibit potent antioxidant activity in various test systems (*9*). Apart from the antioxidant effects, very little is known about other anticancer properties including the antimutagenic and antiproliferative activities of rooibos tea. At present it is not known whether processing of rooibos would adversely affect the biological properties of rooibos. The polyphenolic content and composition of the unfermented rooibos differs from that of the fermented (processed) rooibos (*7*). Von Gadow et al. (*10*) demonstrated that aqueous extracts prepared from processed rooibos were less effective as antioxidants than extracts of unprocessed rooibos, but the effect of the various processing steps on its antioxidant activity was not investigated. The decrease in antioxidant activity was partly attributed to the oxidation of aspalathin (*9*). The processing steps entail "heap fermentation" of the comminuted leaves and fine stems for several hours during which the desired oxidative changes take place, followed by sun-drying and sieving to remove coarse material consisting mainly of woody stems. The dry product is sterilized by steam pasteurization before bulk packaging (*11*).

The aim of this study was to determine the antimutagenic and antioxidant potentials during the different stages of rooibos processing. The *Salmonella* mutagenicity assay was used to determine antimutagenic activity, whereas scavenging of superoxide anion radi-

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cals and hydrogen donating ability toward the stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH•) were used to evaluate the antioxidant activity of aqueous extracts of rooibos collected at the different processing stages.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from Sigma Chemical Co., St Louis, MO [2-acetylaminofluorene (2-AAF), α,α-diphenyl-β-picrylhydrazyl radical (DPPH•), phenazine methosulfate (PMS), nicotinamide dinucleotide phosphate (NADH)] and Roche Pharmaceuticals, Johannesburg, South Africa [nitroblue tetrazolium (NBT)]. All other chemicals and solvents were of analytical reagent grade. The water used for antioxidant testing was treated with a Modulab water purification system (Separations, Cape Town, South Africa). This consisted of a 5 μm filter for the removal of coarse particles, followed in sequence by a carbon filter, a reverse osmosis system, a mixed bed deionizer to obtain water at a conductivity level of 0.1 μs/cm, and finally a filter (0.22 μm) for the removal of microorganisms.

Collection of Tea Samples. Ten random samples (∼1 kg) from each of the five major rooibos processing stages were collected from a rooibos processing plant (Rooibos Ltd., Clanwilliam, South Africa) during February 1998. The plant material consisted of the complete plant, including the stems and leaves, 30 cm above the soil level. Sampling of unfermented samples took place before comminution (∼3−4 mm lengths) to prevent oxidative changes. These samples were dried in a dehydration tunnel at 40 °C for 48 h. After comminution, the tea was placed in heaps on outside platforms, wetted and bruised to initiate "fermention", and fermented for ∼12 h. Fermented rooibos teas were sampled from the heaps after overnight fermentation and dried in a dehydration tunnel at 40 °C for 24 h within 3 h of collection. Subsequent samples were also collected after the tea was sun-dried (∼12 h), after sieving (<10 mesh), and after steam pasteurization (≥90 °C for 2 min).

Preparation of Aqueous Tea Extracts. All samples were pulverized in a laboratory hammermill (serial no. 401 Scientific RSA). The aqueous extracts were prepared by boiling 100 g of material in distilled water (1000 mL) for 30 min. The extracts were filtered twice with a Büchner filter, first through a fine 125 μm mesh cloth (Polymer PES D25/35 supplied by Swiss Silk Bolting Cloth Mfg. Co. Ltd., Zurich, Switzerland) and then through Whatman No. 1 filter paper. The filtrates were frozen (−20 °C) and freeze-dried (Atlas commercial freeze-drier, Copenhagen, Denmark; shelf temperature = 40 °C). Prior to the antimutagenicity testing 2.5% (w/v) solutions of the different freeze-dried tea extracts were prepared in boiled, distilled water. The solutions were centrifuged at low speed (200 rpm) for 30 min at room temperature, and the supernatants were filter under vacuum through a 0.45 μm acetate membrane filter (Micron Separations). Finally, the solutions were filter sterilized through a 0.22 μm acetate syringe filter. The original extract was diluted 2 and 5 times while 0.1 mL was incorporated in the *Salmonella* assay. All of the solutions were freshly prepared prior to the assay and tested in triplicate.

Aqueous extracts for antioxidant testing were prepared from the same unfermented, fermented, sun-dried, and steam-pasteurized samples, and seven samples of each of these processing stages were randomly selected. The sample collected after the sieving process was not included in this assay. The soluble solids content of the extracts was determined gravimetrically on 20 mL aliquots, and the remainder of the extracts was frozen at −20 °C until used for analysis. The total polyphenol concentration of the aqueous extracts was determined according to the method of Singleton and Rossi (*12*). The reaction mixture was incubated at 30 °C for 2 h to allow color development, after which time absorbance was read at 765 nm. Gallic acid was used as standard, and results were expressed as the gallic acid equivalents (GAE) of soluble solids (%, m/m) in the extract.

Antimutagenicity Testing. The protective effect of the different aqueous extracts against mutagenesis in the *Salmonella* test was performed by using the standard mutagenic protocol described by Maron and Ames (*13*). *Salmonella* tester strain TA 98 was used in all of the assays utilizing 2-acetylaminofluorene as the diagnostic mutagen. The assay was performed in the presence of Aroclor 1254 induced S-9 liver homogenate (0.72 nmol of cytochrome P450/mg of protein) prepared from male Fischer 344 rats as described by Maron and Ames (*13*). The S-9 preparation was incorporated in the S-9 mixture at a level of 0.04 mL (2 mg of protein) of S-9/mL of S-9 mixture. An overnight culture of *Salmonella* TA 98 in Oxoid broth 2 was used, and for the standard plate incorporation assay, 0.1 mL of the bacterial tester strain was added to 2 mL of top agar at 45 °C. The mutagen (0.1 mL), tea extract (0.1 mL), and S-9 mix (0.5 mL) were added to the reaction mixture, and the contents were vortexed and poured onto the minimal glucose agar plate. The entire test was perfomed in triplicate, and the plates were incubated in the dark at 37 °C for 48 h. In addition to the test plates, control plates of bacteria without the tea extract, 2-AAF, and S-9 mix were included.

Hydrogen Donating Ability of Aqueous Extracts. The effect of processing on the hydrogen donating ability of the aqueous extracts was quantified in terms of their ability to scavenge DPPH•. Scavenging of DPPH• was determined according to a slightly modified version of the method of Brand-Williams et al. (*14*). An appropriate dilution series (0−0.3 mg of soluble solids/mL) was prepared for each aqueous extract in purified, deionized water, and 0.1 mL of each dilution was added to 4 mL of a 3.04×10^{-5} M methanolic solution of DPPH•, followed by vortexing. The reaction was allowed to take place in the dark for 2 h (at room temperature) to reach steady state conditions, after which time the absorbance was read at 515 nm to determine the concentration of remaining DPPH•. The concentration of remaining DPPH• was used to calculate the EC_{50} values, that is, the amount of antioxidant required to scavenge 50% of the initial DPPH• in the reaction mixture. Lower EC_{50} values indicate higher hydrogen donating efficacy. All tests were done in duplicate and the results averaged.

Superoxide Anion Radical Scavenging Ability. Inhibition of NBT reduction by nonenzymatically generated $O_2^{\bullet-}$ (*15*) was used to determine the $O_2^{\bullet-}$ scavenging ability of the aqueous extracts. An appropriate dilution series (0−0.8 mg of soluble solids/mL) of the aqueous extracts was prepared in purified, deionized water. A 0.5 mL aliquot of each dilution was pipetted into a disposable cuvette, followed by the addition of 0.5 mL of NBT (2.52 mM) and 0.5 mL of NADH (624 μM). The reaction was initiated by the addition of 0.5 mL of PMS (120 μM) and monitored at 560 nm over a period of 2 min (20 readings/min) on a Beckman DU 65 UV−vis spectrophotometer (1 cm path length) with Data Capture software. The NBT, NADH, and PMS solutions were prepared fresh each day in a 0.1 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4) and kept on ice for the duration of the experiment. The percentage inhibition at steady state for each dilution was used to calculate the IC_{50} values. This gives the amount of antioxidant required (measured as the concentration of the stock solution added to the reaction mixture) to scavenge 50% of $O_2^{\bullet-}$, with lower values indicating more effective scavenging of $O_2^{\bullet-}$. All tests were done in triplicate and the results averaged.

Statistical Analyses. One-way ANOVA was performed separately on data obtained for testing of the antimutagenic and antioxidant activities to determine whether the means for test samples differed significantly. Student's *t* test was also performed on the data to identify the significantly different groups within the samples. The differences between the percentage inhibitions of the AAF-induced mutagenic response effected by the different tea samples were analyzed by the nonparametric Wilcoxon signed rank test, assuming that each pair of observations is independent of the other pairs.

RESULTS AND DISCUSSION

Antimutagenicity of Tea Samples. All of the tea preparations significantly protect against mutagenesis

Table 1. Protective Effect of Aqueous Tea Extracts Prepared from Rooibos Tea Sampled from Five Different Processing Stages

treatment	extract[a] (0.02 mL)[b]	extract[a] (0.1 mL)[b]
control (DMSO)	31.72	nd
control (2-AAF)	268.55a	nd
unfermented	166.65c	61.28d
fermented[c]	229.04b	127.43c
sun-dried[d]	241.89ab	141.99bc
sieved	249.43ab	154.42b
steam pasteurized	259.72a	160.78b

[a] The mutagenicity assay was performed using 2-AAF as the mutagen in the presence of metabolic activation. Mean values followed by the same letter do not differ significantly. Each data point represents 10 samples per treatment group, each done in triplicate. nd, not determined. [b] Volume extract per plate. [c] Fermented followed by artificial drying. [d] Fermented followed by sun-drying.

induced by 2-AAF (Table 1) and hence exhibit properties similar to those of the green and black tea preparations (*16*, *17*). Both concentrations of the tea extracts tested showed this protective effect in a typical dose−response manner. The unfermented tea exhibited the highest protective effect, whereas the fermentation process resulted in up to 20% reduction. The drying (∼12 h in the sun) and sieving processes (removal of stems) markedly reduced the protective activity, although the differences were not significant ($P > 0.05$). The pasteurization process has very little effect on the protective effect, indicating that the 2 min treatment at >90 °C did not have a major destructive effect on the antimutagenic property of rooibos. When the percent inhibitions of the 2-AAF positive control by the different tea samples are compared, using a different statistical design of analyses (see Materials and Methods), it becomes apparent that the major reduction in the antimutagenic properties is noticed during the chemical fermentation process (Table 1). However, each processing step significantly ($P < 0.01$) reduced the inhibitory effect of the tea, indicating that each step has an impact on the final antimutagenic potential of the tea. This could provide ample opportunities for the manufacturers to introduce steps circumventing the destruction of the causative principles in the case where rooibos tea is used for the production of neutraceutical products.

Hydrogen Donating Ability of Rooibos Aqueous Extracts. The EC_{50} values for the different processing stages are given in Table 2. The unfermented tea extracts exhibited a higher hydrogen donating ability than extracts prepared from the other processing stages. The hydrogen donating ability of both the ethyl acetate soluble and crude polymeric fractions prepared from the aqueous extract of rooibos decreased as a result of fermentation process (*18*). There was a relatively small, yet significant ($P < 0.05$), difference in the EC_{50} values of the fermented, sun-dried, and steam-pasteurized processing stages, with steam-pasteurized tea more effective than tea from the other two preceding stages.

Superoxide Anion Radical Scavenging Ability. As described above for the hydrogen donating ability, the unfermented tea extract had the highest superoxide anion radical scavenging ability as indicated by the lowest IC_{50} value (Table 2). No significant difference ($P > 0.05$) could be established between the fermented and steam-pasteurized samples, but sun-dried tea was more effective as an $O_2^{\bullet -}$ scavenger than the fermented tea ($P < 0.05$). In contrast to the hydrogen donating ability,

Table 2. Total Polyphenol Content of the Water-Soluble Solids of Rooibos Tea and Their Hydrogen Donating and Superoxide Anion Radical Scavenging Abilities As Affected by Processing

sample	soluble solids[a] (%)	total polyphenols[b] (%)	EC_{50} value[c] (μg/mL)	IC_{50} value[d] (mg/mL)
unfermented	2.31a[e]	40.99d[e]	95.4d[e]	0.177c[e]
fermented[f]	1.63c	34.95b	148.4b	0.242a
sun-dried[g]	1.97b	33.91a	156.6a	0.220b
steam pasteurized	1.84b	35.30c	137.2c	0.223ab

[a] Amount of soluble solids (g) in 100 mL of aqueous extract. [b] Expressed as g of GAE per 100 g of soluble solids. [c] Amount of test sample required (measured as the concentration of the stock solution added to the reaction mixture) to scavenge 50% of 3.0×10^{-5} M DPPH$^{\bullet}$ at steady state conditions. [d] Amount of antioxidant required (measured as the concentration of the stock solution added to the reaction mixture) to scavenge 50% of $O_2^{-\bullet}$. [e] Means in a column followed by a different letter differ significantly ($P \leq 0.05$). [f] Fermented followed by artificial drying. [g] Fermented followed by sun-drying.

any qualitative changes in phenolic composition of the tea that occur in subsequent processing stages after the initial fermentation process have very little effect on the $O_2^{\bullet -}$ scavenging ability of the extracts.

The higher hydrogen donating and $O_2^{\bullet -}$ scavenging abilities of the unfermented tea extract correlate with the higher total polyphenol content as compared to the fermented, sun-dried, and pasteurized tea extracts (Table 2). Processing decreased the total polyphenol content of the soluble solids. Fermentation resulted in a substantial decrease in the dihydrochalcones, aspalathin and nothofagin, with the fermented rooibos containing ∼7% of the dihydrochalcones originally present in the unfermented rooibos (*7*). The ratio of dihydrochalcones to other flavonoids such as rutin, quercetin, isoquercitrin, orientin, iso-orientin, and vitexin also decreases with processing (*7*). Sun-drying of the tea allows further oxidation of polyphenols, especially during slow drying, with subsequent lowering of the hydrogen donating ability of the tea (higher EC_{50} value) compared with the tea that was not sun-dried. Koeppen and Roux (*19*) postulated that aspalathin is converted to its corresponding flavanones, which then form unknown brown products on prolonged exposure to sunlight. Dihydrochalcones are more effective as antioxidants than their corresponding flavanones (*20*), which could offer an explanation for this decrease in hydrogen donating ability with fermentation. However, according to the EC_{50} values, steam pasteurization improved the DPPH$^{\bullet}$ scavenging ability and thus hydrogen donating ability of rooibos. Removal of woody stems with a low water-soluble phenolic content (*21*) before pasteuriztion could explain the higher hydrogen donating ability of steam-pasteurized tea in comparison with the sun-dried tea. However, the total polyphenol content of the sun-dried and steam-pasteurized rooibos extracts did not differ significantly ($P > 0.05$) (Table 2). This could be attributed to large variation in the individual samples, considering that the tea, processed daily at the plant, originates from different plantations (age, locations, etc). Qualitative differences in phenolic content due to removal of woody stems could also explain the efficacy of steam-pasteurized tea in comparison to fermented (tunnel-dried) tea as hydrogen donor and $O_2^{\bullet -}$ scavenger. On the basis of the results of Von Gadow et al. (*22*), a small increase in antioxidant activity due to the heating of rooibos during steam pasteurization should also be considered.

The results from the *Salmonella* test indicated that the samples tested exhibit antimutagenic potential in a typical dose-dependent manner (Table 1). As discussed above, the antimutagenic potential was significantly reduced during the manufacturing process and more specifically during the fermentation stage. Oxidation of polyphenolic compounds during fermentation (*7*) may result in products with lower antimutagenic potential than the unoxidized compounds. Manipulation of the processing of rooibos may reduce the degree of oxidation and hence is likely to affect the antimutagenic potential. This aspect must be further investigated, as flavor will be negatively affected with incomplete fermentation (*23*). As discussed for the hydrogen donating ability of the processed tea, the subsequent steps following the chemical fermentation process also significantly ($P < 0.01$) reduced the inhibitory effect against 2-AAF-induced mutagenesis.

The present study indicated that the reduction in the tea polyphenolic content during fermentation is associated with the decrease in antioxidant and antimutagenic effects. At present it is not known whether the compounds responsible for the antimutagicity are similar to those exhibiting the antioxidant properties. However, a recent study suggested that the antimutaganic activity of aqueous extract from fruits and vegetables could be attributed to their antioxidant activity (*24*). Yen and Chen (*2*) correlated the antioxidant effect of tea extracts to their antimutagenicity, although it varies with the mutagen used and the antioxidant properties. The present data also confirmed this aspect as differences exist in the antioxidant potency of the different samples depending on the method used to detemine the antioxidant potential. Further investigations regarding the isolation and identification of the antimutagenic and antioxidant properties are required to determine whether the same of different compounds are involved in these protective mechanisms.

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