Effect of Extraction Time and Additional Heating on the Antioxidant Activity of Rooibos Tea (*Aspalathus linearis*) Extracts

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The antioxidant activity of aqueous extracts of rooibos tea (*Aspalathus linearis*) as affected by extraction time and additional exposure to heat was determined using the Rancimat and β-carotene bleaching methods. Two fractions of the aqueous extract, consisting of phenolic compounds soluble in ethyl acetate and, after removal of these substances, the remaining polymeric fraction only soluble in water, were analyzed for antioxidant activity. The effect of concentration of the soluble solids of rooibos tea on the oxidation of lard and linoleic acid were assessed by the Rancimat and β-carotene bleaching methods, respectively. Increasing extraction time resulted in increasing induction time for a fixed ratio of soluble solids to lard. Additional heat treatment of tea extracts also increased antioxidant activity according to the Rancimat method, without exhibiting a prooxidative effect at high concentrations. Antioxidant activity of rooibos was demonstrated for both the ethyl acetate soluble phenolic compounds and the highly polymerized polyphenols only soluble in water.

Keywords: *Antioxidant; rooibos; extraction time; heat treatment; Rancimat; β-carotene bleaching*

INTRODUCTION

Rooibos tea is made from the leaves and fine stems of *Aspalathus linearis*, a leguminous shrub indigenous to the Cedarberg mountains of the Western Cape in South Africa. The infusion is often prescribed for calming digestive disorders, reducing nervous tension, and alleviating allergies (Morton, 1983). It was shown to be effective against various dermatological diseases (Shindo and Kato, 1991). The health-giving properties of rooibos tea are attributed to its antioxidant properties (Yoshikawa *et al.*, 1990; Ito *et al.*, 1991). The caffeine-free infusion has a low tannin content (Morton, 1983), but contains a number of phenolic acids (caffeic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, vanillic acid) and flavonoids (aspalathin, (+)-catechin, isoquercitrin, luteolin, quercetin, rutin, vitexin) (Rabe *et al.*, 1994). Most of these compounds are widely distributed in nature and have been shown to possess antioxidant properties (Pratt and Hudson, 1990; Ho *et al.*, 1992; Onyeneho and Hettiarachchy, 1992; Kanner *et al.*, 1994). Von Gadow *et al.* (1997) demostrated antioxidant activity for aspalathin, a dihydrochalcone unique to rooibos tea.

The infusion of rooibos tea is drunk either hot or cold as a substitute for black tea (*Camellia sinensis*). It is occasionally also used as a food ingredient in products such as soups and meat marinades. The consumption of rooibos tea is increasing, not only in South Africa, but also internationally. Other uses of rooibos tea extracts include cosmetic and therapeutic products. Extraction of the water soluble solids of rooibos tea and their phenolic content depend on the time and temperature of extraction (Joubert, 1990a,b). Prolonged extraction at high temperatures is needed to obtain maximum yields of soluble solids (Joubert and Hansmann, 1990).

Aspects which have hitherto not been investigated are the antioxidant activity of rooibos tea fractions (ethyl acetate soluble and the polymeric fraction insoluble in ethyl acetate) and the effect of extraction time and continued heat exposure after extraction on the antioxidant activity of rooibos tea.

MATERIALS AND METHODS

Plant Material and Chemicals. Rooibos tea was supplied by Rooibos (Pty) Ltd, Clanwilliam, South Africa. Tween 40, *trans*-β-carotene, and linoleic acid (approximately 99% purity) were purchased from Sigma Chemical Co., St Louis, MO. Lard was purchased from Eskort Bacon Co-operative Ltd, Natal, South Africa.

Preparation of Water Extracts To Determine the Effect of Extraction Time. Water extracts of rooibos tea were prepared by pouring boiling distilled water on the leaves (120 g/1000 mL), followed by steeping on a boiling water bath for 5, 10, 15, 20, 25, and 30 min, respectively. The extracts were strained and filtered through Whatman No. 54 filter paper using a Buchner filter under vacuum. The filtrate was cooled to room temperature immediately by being placed in an ice bath. The total polyphenol, flavonoid, and total soluble solids contents of the extracts were determined as described below. Aliquots of the extracts were kept frozen (−18 °C) until further use. Preparation of the extracts was done in triplicate. The soluble solids of the extracts before fractionation were designated the total water soluble solids (TWSS).

Preparation of Water Extracts To Determine the Effect of Additional Heating. An extract of rooibos tea was prepared by extracting 100 g of tea with 1000 mL of boiling distilled water on a boiling water bath for 5 min followed by straining, filtering, and cooling. The TWSS of the extract was determined as described below. Aliquots of the extract were then boiled under reflux for 5, 15, and 30 min, respectively. Seven replicates of each heat treatment were prepared. Aliquots were stored frozen (−18 °C) until further use.

Preparation of Water Extracts To Determine the Effect of Concentration of Tea Solids. A concentration series consisting of 50, 200, 400, 600, and 800 mg of soluble tea solids per 100 mL of water was prepared of the TWSS and polymeric water soluble phenols (PWSP, see below), respectively. An aliquot of the water extract, prepared by steeping for 30 min, was used to give the required dilution series of TWSS.

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Preparation of Ethyl Acetate Soluble Fraction. A water extract of rooibos tea was prepared as described above, except that 120 g of tea was steeped in 1000 mL of boiling distilled water for 60 min. Liquid−liquid extraction was carried out with ethyl acetate under reflux for 16 h. The ethyl acetate extract was evaporated under vacuum to dryness with a Büchi Rotavapor (Switzerland), and the residue was stored in a desiccator in the dark until further analysis. The residue was redissolved in methanol for the determination of antioxidant activity.

Preparation of the Polymeric Water Soluble Phenolic Fraction. The water soluble solids of rooibos containing no polyphenols soluble in diethyl ether and ethyl acetate but mainly polymeric phenolic substances were prepared and supplied in kind collaboration by Prof. D. Ferreira, Research Unit for Polyphenol and Synthetic Chemistry, University of the Orange Free State, Bloemfontein, South Africa. Preparation entailed the extraction of a water extract of rooibos tea leaves with diethyl ether and ethyl acetate until 2D paper chromatograms showed complete and exhaustive extraction of flavonoids and phenolic acids (Rabe *et al*., 1994). The residue was freeze-dried and designated the polymeric water soluble phenols (PWSP).

Determination of Total Polyphenol, Flavonoid, and TWSS Contents of Water Extracts. Polyphenol analysis of extracts according to Singleton and Rossi (1965) using the spectrophotometric Folin−Ciocalteu method before and after precipitation of flavonoids (Kramling and Singleton, 1969) gave total polyphenol, non-flavonoid phenols and, by difference, flavonoid contents of the extracts. Results were expressed as gallic acid equivalents (GAE) per 100 g of water soluble solids. The TWSS content of the extract was determined gravimetrically in duplicate. An aliquot (20 mL) was evaporated to dryness on a steam bath followed by further drying overnight under vacuum at 70 °C.

Determination of Antioxidant Activity with the Rancimat Method. The Rancimat method was used to determine the effect of extraction time, additional heating, and concentration of soluble solids (PWSP) on antioxidant activity. The ethyl acetate soluble fraction was also tested with the Rancimat method. The antioxidant activity of tea extracts was determined with slight modifications according to the induction period method of Ho *et al*. (1992). A Rancimat Model 679 (Metrohm AG, Switzerland) was used for the oxidation of lard with and without addition of tea extract. Oxidation was carried out at 90 °C with an air-flow rate of 20 L/h. The soluble solids contents of the extracts were standardized to give 500 mg of soluble solids/100 mL of water for all experiments except when noted otherwise. The lard (2.5 g) was melted for 3 min at 90 °C, and the standardized extract (100 μL) was added to the lard in the reaction vessel, giving a final concentration of 0.02% (mass/mass) of soluble solids, followed by addition of absolute ethanol (1 mL). The mixture was vortexed (Vortex-Genie 2, Scientific Industries) for 8 s before commencement of the test. The effect of concentration was tested using the dilution series of the PWSP described above to give concentrations of 0.002%, 0.008%, 0.016%, 0.024%, and 0.032% (mass/mass) in the lard. At least 20 duplicate determinations were conducted on each sample and the control. The protection factor (PF) was calculated from the induction times with the following formula (Weng and Gordon, 1992):

$$PF = (IP_{lard+additive} - IP_{lard})/IP_{lard} \quad (1)$$

where

$IP_{lard+additive}$ = induction period of lard with additive

IP_{lard} = induction period of pure lard

Determination of Antioxidant Activity with the β-Carotene Bleaching Method. Antioxidant activity of the tea extracts was also determined using a modified version of the β-carotene bleaching method of Pratt (1980). β-Carotene (0.1 mg) was added to a boiling flask together with linoleic acid (20 mg) and Tween 40 (100 mg), all dissolved in chloroform. After evaporation to dryness under vacuum at room temperature with a rotary evaporator, oxygenated distilled water (50 mL) was added and the mixture was emulsified for 1 min with a B15 Branson sonifier cell disruptor at 16% duty cycle of a continuous output control of 3 to form emulsion A. Sonification of the emulsion was done to improve its stability at high temperatures (Frankel *et al.*, 1994). Tea extract (200 μL) containing 500 mg of soluble solids/100 mL of water was pipetted into screw-capped vials to which 5 mL of emulsion A were added. A control consisting of 200 μL of water and 5 mL of emulsion A was prepared. A second emulsion (B), consisting of 20 mg of linoleic acid, 100 mg of Tween 40, and 50 mL of oxygenated water, was also prepared. Water (200 μL) to which 5 mL of emulsion B was added was used to zero the spectrophotometer. Blanks of the tea extract were prepared using emulsion B instead of A. Readings of all samples were taken immediately ($t = 0$) and at 15 min intervals for 2 h ($t = 120$) on a Beckman DU-65 spectrophotometer at 470 nm. The vials were placed in a water bath at 50 °C between measurements. All determinations were performed in duplicate. The effect of concentration of the TWSS and the PWSP on antioxidant activity was tested using a concentration range of 50−800 mg of solids/100 mL of extract. The antioxidant activity coefficient (AAC) was calculated from the data with the following formula (Mallet *et al*., 1994):

$$AAC = 1000[(A_{E(120)} - A_{C(120)})/(A_{C(0)} - A_{C(120)})] \quad (2)$$

where

AAC = antioxidant activity coefficient ranging from 0 to 1000

$A_{E(120)}$ = absorbance of the extract sample at $t = 120$ min

$A_{C(120)}$ = absorbance of the control at $t = 120$ min

$A_{C(0)}$ = absorbance of the control at $t = 0$ min

Curve Fitting. AAC and induction period values for extract concentrations of 500 mg of soluble solids/100 mL of solution were estimated by curve fitting of data obtained for the different concentration series. Polynomial curves were fitted to the data according to the least squares difference method using Turbo Pascal numerical methods (Borland International Inc., Version 4.0).

Statistical Analysis. ANOVA (SAS Release 6.03) was used to determine whether extraction time and additional heating significantly affect the parameters tested. The Student t least significant difference (LSD) ($P = 0.05$) was calculated to compare means in cases where the F-values showed significant differences. Regression analysis was carried out on data obtained for extraction time to test for significant trends over extraction time.

RESULTS AND DISCUSSION

Effect of Extraction Time on Antioxidant Activity. The antioxidant activity of the total water soluble solids (TWSS) of rooibos tea increased with increasing extraction time as indicated by the induction times and PF obtained using the Rancimat method (Table 1). Induction periods differed significantly ($P = 0.0001$) and increased significantly ($P = 0.0001$) with extraction time. The increasing antioxidative effect can be attributed to compositional changes of the TWSS since a fixed ratio of TWSS to lard was used. Extraction of small molecules and highly soluble substances would be favored initially, due to a high rate of diffusion. Large molecules like proanthocyanidins in rooibos tea (Ferreira *et al.*, 1995) and less soluble substances would diffuse at a slower rate from the leaves (Joubert, 1990b). This was, however, not reflected by the phenolic content

Table 1. Effect of Extraction Time on Antioxidant Activity of Rooibos Tea As Assessed by the β-Carotene Bleaching and Rancimat Methods

extractn time (min)	AAC[a]	inductn period[b] (h)	PF[c]
control[d]		0.57 ± 0.14[f] a[g]	
5	705.95 ± 34.40[e] ab[g]	1.58 ± 0.37 b	1.77
10	650.96 ± 65.17 bc	1.76 ± 0.54 b	2.09
15	547.62 ± 124.79 c	1.96 ± 0.30 c	2.44
20	807.15 ± 27.57 a	1.97 ± 0.24 c	2.46
25	723.08 ± 84.21 ab	2.10 ± 0.48 cd	2.68
30	605.31 ± 30.99 bc	2.21 ± 0.33 d	2.88
LSD[h] ($P = 0.05$)	131.02	0.1891	

[a] AAC = antioxidant activity coefficient; determined by β-carotene bleaching method; concentration of stock solution = 500 mg of soluble solids/100 mL of solution. [b] Determined by Rancimat method; concentration of stock solution = 500 mg of soluble solids/100 mL of extract [0.02% (m/m) of lard]. [c] PF = protection factor = $(IP_{lard+additive} - IP_{lard})/IP_{lard}$ (Rancimat method). [d] Pure lard. [e] Mean ± standard deviation ($n = 6$). [f] Mean ± standard deviation ($n \geq 20$). [g] Means within a column followed by the same lowercase letter are not significantly different at $P = 0.05$. [h] LSD = least significant difference.

Table 2. Effect of Extraction Time on the Total Water Soluble Solids (TWSS) Content of Rooibos Tea

extractn time (min)	TWSS (g/100 mL)
5	1.76 ± 0.11[a] a[b]
10	2.04 ± 0.03 b
15	2.08 ± 0.03 bc
20	2.27 ± 0.18 d
25	2.19 ± 0.01 c
30	2.42 ± 0.13 e
LSD[c] ($P = 0.05$)	0.1133

[a] Mean ± standard deviation ($n = 3$). [b] Means within a column followed by the same lowercase letter are not significantly different at $P = 0.05$. [c] LSD = least significant difference.

of the TWSS. The extract concentration (TWSS) increased significantly with extraction time ($P = 0.0001$) (Table 2), but its total polyphenolic and flavonoid content remained constant at 30.44% and 20.53%, respectively. Qualitative changes in phenolic composition would not necessarily be reflected by the Folin−Ciocalteu method due to its lack of sensitivity to differentiate between specific phenolic compounds.

Results obtained with the β-carotene bleaching method did not indicate a great difference in the inhibition of β-carotene bleaching between different extraction times (Figure 1). The extracts were effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β-carotene in comparison with the control. Although there was a significant difference in antioxidant activity coefficients (AAC) between the samples ($P = 0.0159$), no significant trend as a result of extraction time ($P > 0.05$) (Table 1) was obtained. The AAC of all the extracts was high (540 to about 800), indicating marked antioxidant activity for the tea extracts at the concentrations of TWSS tested (Table 1).

Rooibos tea is traditionally brewed for long periods of time. The results obtained with the Rancimat method (Table 1) showed that this practice does have merit with regard to antioxidant activity. Extraction times of longer than 30 min could be looked at in a future project to determine their effect on antioxidant activity.

Effect of Additional Heating of Extracts on Antioxidant Activity. The results from the β-carotene bleaching method (Table 3) did not show any significant difference in antioxidant activity with longer heating

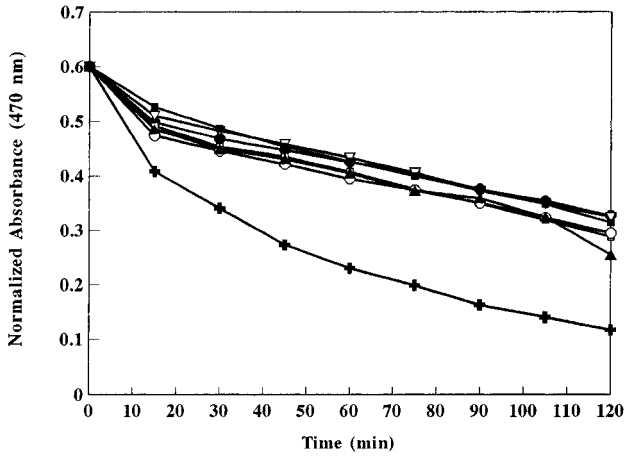

Figure 1. Effect of extraction time on antioxidant activity of rooibos tea extracts as assessed with the β-carotene bleaching method. Stock solutions of tea solids (TWSS) contained 500 mg of soluble solids/100 mL of water: (+) control; (■) 5 min; (▲) 10 min; (○) 15 min; (□) 20 min; (▽) 25 min; (●) 30 min.

Table 3. Effect of Heating Time of Rooibos Tea Extracts on Antioxidant Activity As Assessed by the β-Carotene and Rancimat Methods

heating time (min)	AAC[a]	inductn period[b] (h)
control[c]		0.79
5	590.99 ± 43.23[d] a[e]	1.95 ± 0.49[f] a[e]
15	646.24 ± 91.64 a	2.26 ± 0.47 ab
30	584.16 ± 63.04 a	2.46 ± 0.37 b
LSD[g] ($P = 0.05$)	77.55	0.21

[a] AAC = antioxidant activity coefficient; determined by β-carotene bleaching method; concentration of stock solution = 500 mg of soluble solids/100 mL of solution. [b] Determined by Rancimat method; concentration of stock solution = 500 mg of soluble solids/100 mL of solution [0.02% (m/m) of lard]. [c] Pure lard. [d] Mean ± standard deviation ($n = 4$). [e] Means within a column followed by the same lowercase letter are not significantly different at $P = 0.05$. [f] Mean ± standard deviation ($n \geq 20$). [g] LSD = least significant difference.

times of the tea extracts. However, the induction times as measured with the Rancimat method did show a statistically significant difference ($P < 0.05$) with heating time (Table 3). The antioxidant activity of the TWSS of rooibos tea increased as the heating time was lengthened (Table 2). Part of the traditional preparation of the tea entails brewing on the stove for an extended time (Joubert, 1990c). This practice could therefore affect the antioxidant activity of extracts favorably due to the prolonged exposure to heat.

Effect of Concentration of Soluble Solids on Antioxidant Activity. The effect of the concentration of TWSS and PWSP on antioxidant activity was tested using the Rancimat and β-carotene bleaching methods (Figures 2 and 3). In the case of the Rancimat method (Figure 2), the maximum level of antioxidant tested was 60% higher than the maximum concentration of 0.02% (m/m) of the lard as allowed by law in foodstuffs in the United States (Specchio, 1992). Figure 2 shows the increase in induction time with increasing concentration of the PWSP as determined by the Rancimat method. The increase in induction time with an increase in solids concentration from 0.008% to 0.032% was only slightly less than the increase from 0% to 0.008%. The increase in the AAC value with increasing concentration of TWSS, as well as PWSP, followed a trend similar to that of the induction time, although the increase in AAC value with concentration was more gradual than in the case of induction time. No prooxidative tendency was

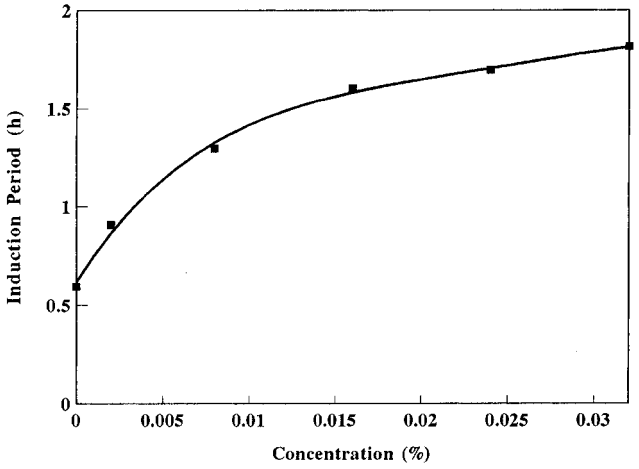

Figure 2. Effect of the concentration of polymeric water soluble phenols (PWSP) of rooibos tea on the induction period of lard as assessed by the Rancimat method.

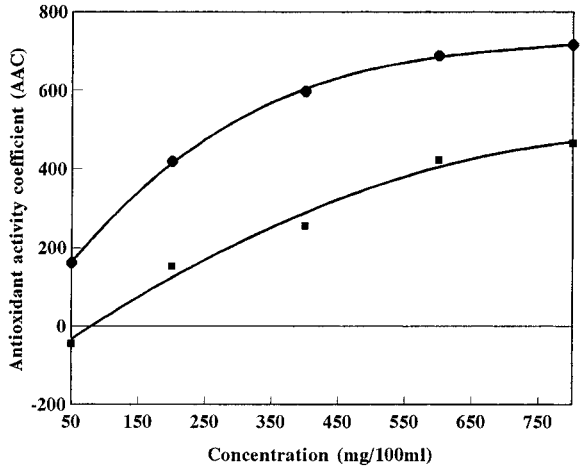

Figure 3. Effect of the concentration of total water soluble solids (TWSS) (●) and polymeric water soluble phenols (PWSP) (■) on antioxidant activity of rooibos tea as assessed with the β-carotene bleaching method.

found with either method at the concentration of soluble solids used. Additional testing at even higher concentrations is needed to ascertain whether the tea could become prooxidative at a higher concentration.

Effect of Fractionation of the Water Soluble Solids on Antioxidant Activity. Higher AAC values were obtained for the TWSS than for PWSP (Figure 3), indicating that extraction of the TWSS with diethyl ether and ethyl acetate removed substances that contributed to antioxidant activity. This was substantiated by analysis of the antioxidant activity of the ethyl acetate soluble solids (Table 4). The protection factor (PF), which takes the different controls into account, and the AAC value obtained for the ethyl acetate soluble solids were higher than in the case of PWSP. The ethyl acetate soluble solids are, therefore, more effective antioxidants than PWSP on a mass basis. Extraction of TWSS with diethyl ether and ethyl acetate removes the phenolic acids and flavonoids such as caffeic acid, ferulic acid, protocatechuic acid, rutin, luteolin, and quercetin (Rabe *et al.*, 1994). These substances have been demonstrated to be potent antioxidants (Torel *et al.*, 1986; Brand-Williams *et al.*, 1995). This indicates the importance of fractionation or selective extraction when aiming to maximize the antioxidant activity potential of rooibos tea solids on a mass basis.

The PF of the ethyl acetate fraction of rooibos tea is approximately 4−5 times that of the PWSP (Table 4). It is also 2−3 times that of the TWSS for extraction times longer than 10 min (Table 1). The AAC values given in Table 4 show that the ethyl acetate extract is about 1.5 times more potent than the PWSP, but the TWSS fraction has a higher AAC value than that of the ethyl acetate fraction (Table 4). On the basis of results obtained with the Rancimat, it would have been expected that the AAC value for ethyl acetate soluble solids would be higher than that of the TWSS. A possible explanation for this discrepancy could be the intrinsic character of the medium in which the test was performed. The ethyl acetate soluble solids are lipophilic and thus displayed a higher antioxidant activity than the predominantly hydrophilic TWSS in the lard (Rancimat method) probably due to the poor solubility of the TWSS in lard. However, in the oil-and-water emulsion used in the β-carotene bleaching method, solubility of the TWSS would be enhanced, explaining the higher antioxidant activity obtained compared with that of the ethyl acetate soluble solids. It could therefore be deduced that the non ethyl acetate fraction makes an important contribution to the antioxidant activity of rooibos tea and deserves further research concerning its composition. The highly polymerized polyphenols of the water extract of rooibos tea have not yet been characterized.

Table 4. Antioxidant Activity of Ethyl Acetate Soluble Solids (EASS) and Polymeric Water Soluble Phenols (PWSP) of Rooibos Tea As Assessed by the β-Carotene Bleaching and Rancimat Methods

fraction	AAC[a]	inductn period (h)	inductn period (control)[c] (h)	PF[d]
EASS	559.53	3.69	0.56	5.59
PWSP	353.35[e]	1.36[f]	0.62	1.19

[a] AAC = antioxidant activity coefficient; determined by β-carotene bleaching method; concentration of stock solution = 500 mg of soluble solids/100 mL of solution. [b] Determined by Rancimat method; concentration of stock solution = 500 mg of soluble solids/100 mL of extract [0.02% (m/m) of lard]. [c] Pure lard. [d] PF = protection factor = $(IP_{lard+additive} - IP_{lard})/IP_{lard}$ (Rancimat method). [e] Value estimated with a polynomial least squares function (three terms) from data given in Figure 3. [f] Value estimated with a polynomial least squares function (five terms) from data given in Figure 2.

It can thus be concluded from the results that all extracts of rooibos tea (*A. linearis*) at the concentrations tested have antioxidant activity irrespective of the testing method. The change in antioxidant activity of rooibos tea as a result of extraction and heating time and extraction solvent is important for the preparation of the tea as well as the extraction of antioxidative compounds for use in medicinal preparations.

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