Effect of simulated adult and infant digestion on the antioxidant and cellular protective effects of *Camellia sinensis* and *Aspalathus linearis* tea

by

Petra Müller

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Supervisors: **Prof A. Oelofse**

Prof M.J. Bester

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Declaration

I, Petra Müller declare that the dissertation, which I hereby submit for the degree Magister Scientiae in Nutrition at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

Date: 25 June 2015

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Research contributions

Certain sections of this Masters Dissertation have been presented at the International Union of Food Science and Technology (IUFoST) 2012 Brazil Congress at Foz do Iguaçu, 5-9 August. The following abstracts were accepted and presented as a poster and oral presentation, respectively. These abstracts, as accepted, are attached in the Addendum.

Poster Presentation:

A comparison of total antioxidant content and activity of 4 commercially available South African teas and their protection against oxidative damage.

Oral Presentation:

Effect of digestion of *Camellia sinensis* and *Aspalathus linearis* teas on antioxidant content and activity and their protection against oxidative damage

Abstract

Background: Many South Africans have a poor antioxidant status and fermented *Camellia sinensis* (BT) and *Aspalathus linearis* (RT) teas that are widely consumed by adults and infants is a possible source of antioxidants which can address this deficiency by protecting cells and tissue against reactive oxygen species damage.

Objective: The aim of this study was to determine the extent to which antioxidant properties of these teas are affected by adult and infant gastrointestinal digestion.

Methods: Water extracts of BT (n = 4) and RT (n = 3) were prepared and were subjected to adult and infant simulated *in vitro* digestion. For pH controls, stomach (SD) and subsequent duodenal digestion (SDD) fractions the total polyphenolic (TPC) and flavonoid (TFC) content was determined. Antioxidant activity was determined with the DPPH, TEAC and ORAC assays. The NO scavenging ability of each fraction as well as the cellular antioxidant activity in the Caco-2 cell line using the DCHF-DA assay was evaluated.

Results: With adult and infant digestion these antioxidant properties in the SD and SDD phase of digestion were increased, unchanged or reduced due to pH dependent extraction or degradation of polyphenolics and/or the biotransformation of flavanols and other flavonoids. TFC of BT remained relatively stable with infant and adult digestion, as well as with infant digested RT. Significant losses in antioxidant activity as determined by the DPPH and ORAC assays of BT and RT were found following complete adult and infant digestion, whereas TEAC assay did not show such a great loss following digestion. NO scavenging assay and DCFH-DA were less affected by infant digestion than by adult digestion. Following infant digestion of BT and RT, cellular protection against oxidative damage was 94.97% and 83.99%, respectively. For adult digestion, cellular protection was 83.15% and 71.71% for complete digested BT and RT,

respectively. The RSC of tea was calculated and it was found that one cup BT and two cups RT provided the equivalent of 200 mg vitamin C for adults. One cup of both teas provided more than the equivalent of 30 mg vitamin C needed for infants. GIT enzymes may serve as a protein matrix that protects polyphenols against pH driven degradation or quench radicals.

Conclusion: Adult and infant digests of fermented BT with in most instances was found to have better antioxidant properties than RT. As a significant amount of antioxidant activity remained following digestion, both RT and BT may assist in the prevention and management of infections and chronic diseases. However BT contains caffeine which makes RT, the better choice for infants, children and caffeine sensitive adults.

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Abbreviations

ABTS	2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid radical cation
DCFH-DA	2′,7′-dichlorfluorescein-diacetate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	epicatechin
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
ET	electron transfer
FRAP	ferric reducing antioxidant potential
GIT	gastrointestinal tract
HAT	hydrogen atom transfer reaction
$HO^{\bullet}$	hydroxyl radical
LC-MS	liquid chromatography-mass spectrometry
LDL	low-density lipoproteins
$^{\bullet}NO$/NO	nitric oxide
$^{\bullet}NO_2$	nitrogen dioxide
NOS	nitric oxide synthase
$O_2^{-\bullet}$	superoxide radical
$^1O_2$	singlet oxygen
OH	hydroxyl
$O{=}NOO^-$	peroxynitrite
ORAC	oxygen radical absorbance capacity
PUFAs	polyunsaturated fatty acids
RNS	reactive nitrogen species
$ROO^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
SAVACG	The South African Vitamin A Consultative Group
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TFC	total flavonoid content
TPC	total polyphenolic content
VLDL	very low-density lipoproteins

Chapter 1: Introduction, hypotheses and objectives

1.1 Introduction

The South African Vitamin A Consultative Group (SAVACG) (1996) identified marginal vitamin A deficiency as a severe public health problem in South Africa, with 33% of children aged 6-71 months having a serum vitamin A concentration < 20 µg/dl. Labadarios *et al*. (2005) stated that vitamin A, C and E intakes for South African children were below two-thirds of the Recommended Dietary Allowances. These vitamins are also important antioxidants that defend against free-radical damage (Whitney & Rolfes, 2008, p. 391). Vorster *et al*. (1997) noted that fruit and vegetable intake among South Africans is inadequate and therefore it can be assumed that a large percentage of South Africans have inadequate intake of antioxidants through their diets. A low antioxidant status is associated with an increased susceptibility for the development of childhood infectious diseases such as diarrhoea, respiratory infections and measles, as well as non-communicable adult chronic diseases such as cardiovascular disease, diabetes and cancer (Anderson & Chu, 2007). In addition, exposure to environmental pollutants such as metals and toxins through food, beverages and drinking water as well as lifestyle exposure such as tobacco usage and alcohol consumption increases the population's need for antioxidants (Patriarca *et al*., 2000). For many communities the main source of antioxidants (Chun, Chung & Song, 2007; Hertog *et al*., 1993) is tea as it is an affordable, shelf-stable and highly obtainable source of antioxidants.

Tea is the second most popular beverage in the world with an estimated 18-20 billion cups consumed daily (Marcos *et al*., 1998). Green tea is mostly consumed in Asia, particularly in Japan and China, North Africa and the Middle East (Balentine, Wiseman, & Bouwens, 1997). Black tea is typically consumed in the U.S., Europe, Africa and India (Wiseman, Balentine, & Frei, 1997). Tea can be classified as unfermented, semi-fermented, and fermented. Unfermented tea is unwilted and unoxidised, whereas fermented tea is wilted, often crushed, and fully oxidised. Green tea (*Camellia sinensis*) and green Rooibos tea (*Aspalathus linearis*) are examples of unfermented tea (Ferruzzi, 2010). Black tea (*Camellia sinensis*) and Rooibos tea (*Aspalathus linearis*) are examples of fermented tea (Ferruzzi, 2010).

In South Africa, tea is widely consumed by all communities (Labadarios *et al*., 2005). Black tea is commonly consumed in the North-West province (Breet *et al*., 2005), while Rooibos tea is typically consumed in the Western Cape. Tea is also consumed by infants and is often introduced into the diet at as early as 6 months of age (Faber & Benadé, 2007).

Tea is abundant in polyphenolic compounds, particularly flavonoids, which are antioxidants that are believed to offer protection by various mechanisms that involve suppressing reactive oxygen species (ROS), scavenging ROS, and protecting the antioxidant defence systems in the body (Pietta, 2000). The ultimate antioxidant activity of tea radical scavengers is dependent on digestion conditions that results in biotransformation of flavanols and other flavonoids in the stomach, small intestine and colon of the gastrointestinal tract (GIT) (Spencer, 2003).

Although the antioxidant content and activity of *Camellia sinesis* and *Aspalathus linearis* teas have been extensively researched and the active components identified, little is known related to the effects of GIT digestion on activity, especially of Rooibos tea. Knowledge whether there are significant differences between adult and infant digestion of black and Rooibos tea related to antioxidant activity and ability of these teas to protect against oxidative damage is limited.

1.2 Hypotheses

1) The antioxidant content and antioxidant activity, NO scavenging activity and cellular antioxidant activity of locally consumed fermented *Camellia sinensis* and *Aspalathus linearis* teas will contribute significantly to the antioxidant status of the South African diet.

As a result of the oxidation processes of fermented Rooibos tea, lower amounts of flavonoids, particularly aspalathin, resulting in fermented Rooibos tea's antioxidant capacity being lower than that of unfermented Rooibos tea (Standley *et al*., 2001). Similar to Rooibos tea fermentation, changes that occur during the process of fermentation influences the different antioxidant capacity of black and green tea (Pellegrini *et al*., 2003). Flavanols in green tea leaves undergo an oxidative polymerisation by polyphenol oxidase, turning the leaves black. A considerable amount of catechin content of green tea is converted to oxyproducts during oxidation, such as thearubigins and theaflavins, which results in a loss of antioxidant capacity (Pellegrini *et al*., 2003). Therefore, it is expected that green tea and unfermented Rooibos tea will

have higher antioxidant content and activity than black tea and fermented Rooibos tea, respectively, but that both would still provide significant amounts of antioxidant activity.

2) Using an *in vitro* simulated adult digestion model, the measured antioxidant, NO scavenging and cellular protection parameters for fermented *Camellia sinensis* and *Aspalathus linearis* teas will remain stable with stomach digestion and will be reduced with duodenal digestion compared to undigested extracts, and these differences will mainly be due to the effect of pH.

The final antioxidant potential of tea antioxidants is dependent on digestion conditions that results in biotransformation of flavanols and other flavonoids in the stomach, small intestine and colon of the GIT (Spencer, 2003). Acidic pH conditions can decrease antioxidant activity by the degradation of polyphenolics (Record & Lane, 2001), cause the increased extraction of polyphenolics or have no significant effect (Viljoen, 2008; Neilson *et al*., 2007). Numerous studies have observed that tea antioxidants are particularly affected by near neutral conditions (Ferruzzi, 2010; Spencer, 2003; Green *et al*., 2007; Viljoen, 2008). These studies propose that polyphenolic compounds are relatively stable under acidic conditions, however under neutral and alkaline conditions they degrade in pH driven auto-oxidation type reactions. Oxidised products sometimes possess greater radical scavenging activity than their original flavonoids (Spencer, 2003). Antioxidant content and activity should therefore remain stable during stomach digestion and decrease with duodenal digestion.

3) Using an *in vitro* simulated infant digestion model, the measured antioxidant, NO scavenging and cellular protection parameters for *Camellia sinensis* and *Aspalathus linearis* teas will remain stable with SD and will be reduced with SDD, and these differences will also be due to the effect of pH.

Infant digestion differs from adult digestion, in that during stomach digestion in infants, pH is roughly 4 as opposed to pH 2 in adult stomach digestion (Li-Chan & Nakai, 1989). Although generally accepted that polyphenolics are stable at pH 2, little is known regarding the effect of pH 4. Studies have demonstrated that neutral (Ferruzzi, 2010; Spencer, 2003; Green *et al*., 2007; Viljoen, 2008) pH conditions may negatively affect the polyphenolic content and activity of tea. Some studies have also found that acidic conditions have no significant effect on antioxidant

content and activity of tea (Viljoen, 2008; Neilson *et al*., 2007), and that under acidic conditions polyphenolic compounds remain stable. Thus it is expected that the antioxidant content and activity remains stable with stomach digestion and will be reduced with duodenal digestion.

1.3 Objectives

- To determine for fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* teas the total phenolic content, the total flavonoid content, the antioxidant activity and the ability to scavenge NO and protect against oxidative damage in a cellular environment.

- To determine for *Camellia sinensis* and *Aspalathus linearis* teas the effect of adult stomach (SD) and duodenal (SDD) digestion on the antioxidant content, antioxidant activity, and the ability to scavenge NO and protect against oxidative damage in a cellular environment.

- To determine for *Camellia sinensis* and *Aspalathus linearis* teas the effect of infant SD and SDD on the antioxidant content, antioxidant activity, and the ability to scavenge NO and protect against oxidative damage in a cellular environment.

- Finally, to determine whether measured antioxidant activity for *Camellia sinensis* and *Aspalathus linearis* teas and their digests will still contribute significantly to nutritional requirements related to antioxidant intake.

Chapter 2: Literature review

2.1 Introduction

Tea is consumed by most communities in South Africa (Labadarios *et al*., 2005). One of these teas is Rooibos (*Aspalathus linearis*) tea which is a traditional herbal tea of the Khoi-San people of the Cederberg region of the Western Cape (van Wyk, 2008). Dutch settlers to the Cape consumed Rooibos tea as an alternative to black tea, which was expensive and not readily available to the settlers. In the 1930s, Rooibos tea was cultivated and became a commercial crop (South African Rooibos Council, 2014). Today, Rooibos tea is cultivated mainly in the Cederberg mountain area surrounding Citrusdal, Clanwilliam and Nieuwoudtville regions (Joubert *et al*., 2008a). Rooibos tea is marketed as a health beverage as it is naturally caffeine-free, additive-free, preservative-free, colourant-free and very low in tannin. Rooibos tea is often used as a base for exotic, blended herbal teas (Department: Agriculture, Forestry & Fisheries, 2012). Based on its health properties, Rooibos tea is being widely marketed with the development of products such as Rooibos cappuccino, and Rooibos Junior tea for children and Rooibos ice tea, which is a mixture of fruit juice and Rooibos tea. Rooibos is also added to other types of products such as cosmetics. For example, the Annique range of cosmetic products that consists of skincare, body, make-up, and fragrance products. Green Rooibos is a relatively new unfermented Rooibos tea commercial product, the availablity of which is limited and is therefore not widely consumed.

Black tea (*Camellia sinensis*) was brought to South Africa by European immigrants, who traditionally drank tea. Sir Liege Hulett planted imported Assam seed from India on his Kearsney estate near Stanger in 1877 (McIver, 2009). However, the black tea industry died in 1949 as producers in Natal decided on a less labour intensive crop, namely sugar. In 1963 the tea industry became economically viable again, and since then ten estates have been established in the Limpopo Province, Mpumalanga, Kwa-Zulu Natal and Eastern Cape. The industry currently produces roughly 50% of the needs of the subcontinent (McIver, 2009). As for Rooibos tea several tea-based products are also available and these include skincare products and iced tea beverages. Identified ingredients based on antioxidant activity and reported health benefits are also available as nutraceutical products. For example, epigallocatechin gallate (EGCG), a major

component of black tea has been found to assist with the prevention of cardiovascular disorders, reduce bacterial virulence factors, and aid in weight loss (Sajilata, Bajaj & Singhal, 2008).

2.2 Chemical composition of tea

The macronutrients present in fermented black and Rooibos tea is presented in Table 2.1.

Table 2.1: Macronutrient composition of fermented *Camellia sinensis* and *Aspalathus linearis* tea infusions

Macronutrient	Black Tea (g/100 g)	Rooibos Tea (g/100 g)
Carbohydrate	0.3	0.2
Protein	0.0	0.0
Fat	0.0	0.0
Fibre	0.0	0.0

(MRC South Africa, 2011)

Rooibos is naturally caffeine free, and is also a low tannin beverage when compared to *Camellia sinensis* teas (Joubert *et al*., 2008a). The leaf tannin content of fermented Rooibos is 3.2-4.4% (Joubert *et al*., 2008a). Rooibos tea contains several unique flavonoids (Table 2.2) and these include aspalathin, a C – C linked dihydrochalcone glucoside, which is a unique monomeric flavonoid compound found in Rooibos tea (Koeppen & Roux, 1965a; Koeppen & Roux, 1966; Rabe *et al*., 1994), and the cyclic dihydrochalcone aspalanin is currently known to only be isolated from *Aspalathus linearis* (Shimamura *et al*., 2006). Another rare compound found in Rooibos is nothofagin, a 3-dehydroxy dihydrochalcone glucoside that is also present in the heartwood of *Nothofagus fusca* (Hillis & Inoue, 1967).

Other C – C linked β-D-glucopyranosides found in Rooibos include the flavones orientin, iso-orientin (Koeppen & Roux, 1965b), vitexin and isovitexin (Rabe *et al*., 1994), and the flavanones dihydro-orientin, dihydro-iso-orientin (Bramati *et al*., 2002) and hemiphlorin (Shimamura *et al*., 2006). Hot water extracts of unfermented and fermented Rooibos tea contain 15.22% and 2.96% flavonoids, respectively (Joubert *et al*., 2005), and there is a decrease in total phenolic content of the hot water extract of Rooibos with fermentation (Standley *et al*., 2001; Joubert *et al*., 2008b).

Table 2.2: Total polyphenol[a] and major flavonoid[b] content of *Aspalathus linearis* aqueous extracts

Compound	Unfermented Rooibos	Fermented Rooibos
Aspalathin	12.29	0.61
Nothofagin	1.08	0.17
Orientin	0.59	0.76
Iso-orientin	0.81	0.85
Vitexin	0.13	0.17
Isovitexin	0.17	0.11
Isoquercitrin + rutin	0.16	0.11
Total flavonoids	**15.22**	**2.96**
Total polyphenols	**39.30**	**34.25**

[a] *Results expressed as g galic acid equivalents/100 g dried extract determined with Folin-Ciocalteau reagent*
[b] *Results expressed as mass percentage of solids*
(Joubert et al., 2005)

The individual polyphenolics in green and black tea were identified by Stewart, Mullen and Crozier (2005) using liquid chromatography-mass spectrometry (LC-MS) and these are listed in Table 2.3. According to Łuczaj and Skrzydlewska (2005), fresh leaves of black tea contain about 36% polyphenolic compounds on a dry substance mass basis. Catechins are the most abundant polyphenols found in black tea, of which the most significant are epigallocatechin (EGC), EGCG, epicatechin (EC), and epicatechin gallate (ECG) (Łuczaj & Skrzydlewska, 2005). Catechins that are present in smaller amounts include gallocatechin, epigallocatechin digallate, 3-methylepicatechin gallate, catechin gallate, and gallocatechin gallate (Łuczaj & Skrzydlewska, 2005).

Table 2.3: Content of phenolic compounds and purine alkaloids in *Camellia sinensis* teas

	Green Tea (µM)	Black Tea (µM)
Phenolic acids		
Gallic acid	11	183
5-Galloylquinic acid	258	146
Total phenolic acids	**269**	**329**
Flavan-3-ols		
(–)-Gallocatechin	513	n.d.
(–)-Epigallocatechin	1594	48
(–)-Epicatechin	374	34
(–)-Epigallocatechin gallate	1202	52
(–)-Epicatechin gallate	389	58
Total Flavan-3-ols	**4072**	**192**
Caffeoylquinic acids		
3-Caffeoylquinic acid	9	n.d.
5- Caffeoylquinic acid	65	n.d.
Total caffeoylquinic acids	**74**	**0**
Flavonols		
Quercetin rhamnosyl galactoside	3	4
Quercetin-3-rutinoside	24	29
Quercetin-3-galactoside	21	27
Quercetin-hexose-rhamnose-rhamnose	22	25
Kaempferol-rhamnose-hexose-rhamnose	4	5
Kaempferol-galactoside	9	11
Kaempferol-3-rutinoside	16	21
Kaempferol-3-glucoside	29	29
Total flavonols	**128**	**151**
Theaflavins		
Theaflavin	n.d.	117
Theaflavin-3-gallate	n.d.	168
Theaflavin-3′-gallate	n.d.	87
Theaflavin-3-3′-digallate	n.d.	194
Total theaflavins	**0**	**566**
Purine alkaloids		
Caffeine	1194	1295
Theobromine	54	49
Total purine alkaloids	**1248**	**1344**

Results represent concentration of phenolics in a water extract of Finlays green and black tea leaves (3 g/300 mL) (Stewart et al., 2005)

2.3 Antioxidant content and activity of tea

The 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and Trolox equivalent antioxidant capacity (TEAC) assays which are electron transfer assays (ET), as well as the oxygen radical absorbance capacity (ORAC) assay which is a hydrogen atom transfer reaction assay (HAT) (Tabart *et al*., 2009), are the most commonly used assays to measure antioxidant activity. The capacity of an antioxidant to reduce an oxidant is measured by ET-based assays. A colour change occurs when the oxidant is reduced, with the degree of change corresponding to the sample's antioxidant concentration (Zulueta, Esteve & Frígola, 2009). With HAT-based assays, antioxidants and substrates compete for thermally generated peroxyl radicals by means of decomposition of azo-compounds (Zulueta *et al*., 2009).

As a result of fermentation, the antioxidant activity, in terms of $O_2^{-\bullet}$, $DPPH^{\bullet}$, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid radical cation ($ABTS^{\bullet+}$) and ferric reducing antioxidant potential (FRAP) assays (Table 2.4), was higher for unfermented compared to fermented Rooibos hot water extracts (Joubert *et al*., 2008a). This was due to a decrease in total polyphenolic content (TPC) that occurs with fermentation (Joubert *et al*., 2008a).

With fermentation and the resulting oxidation, the flavonoid content of fermented Rooibos tea was reduced particularly for aspalathin, and as a consequence the antioxidant capacity of fermented Rooibos tea was lower than unfermented, green Rooibos tea (Standley *et al*., 2001). Yoo *et al*. (2008) found that, using Chinese hamster lung fibroblast V79-4 cells, fermented Rooibos tea had a protective effect against oxidative stress caused by H_2O_2. Furthermore, in these cells the antioxidant enzymes superoxide dismutase and catalase were induced. A protective effect of Rooibos against lipid peroxidation was demonstrated in linoleic acid emulsions (Joubert *et al*., 2005) and rat liver microsomes (Joubert *et al*., 2008b).

Similar to Rooibos tea, fermentation also influences the different antioxidant capacity of black and green tea (Pellegrini *et al*., 2003). Flavanols in green tea leaves undergo an oxidative polymerisation by polyphenol oxidase, turning the leaves black. A considerable amount of catechin content of green tea is converted to oxyproducts during oxidation, such as thearubigins and theaflavins, and as a result decreases in antioxidant capacity following fermentation (Pellegrini *et al*., 2003). This is supported by the study of Pellegrini *et al*. (2003) where it was

found that green tea had a considerably higher antioxidant activity of 6.01 mmol Trolox/L compared to 3.60 mmol Trolox/L for black tea when determined with the TEAC assay.

Table 2.4: The antioxidant activity of hot water extract of unfermented and fermented *Aspalathus linearis*

Assay	Parameter	Unfermented Rooibos	Fermented Rooibos	% Difference
FRAP	Total antioxidant activity[a]	1.98	1.45	30.90
$ABTS^{\bullet+}$ scavenging	Total antioxidant activity[b]	2.37	1.72	31.78
$DPPH^{\bullet}$ scavenging	% Scavenging[c]	86.6	83.4	3.20
	% Scavenging[d]	87.3	83.0	4.30
	EC_{50}[e]	2.33	3.62	43.36
	EC_{50}[e]	3.24	3.87	17.72
	Rate of scavenging[f]	8.30×10^{-4}	7.35×10^{-4}	12.14
$O_2^{\bullet-}$ scavenging	IC_{50}[g]	44.4	60.5	30.70
	IC_{50}[g]	69.4	78.3	12.05
Linoleic acid emulsion oxidation	% Inhibition (CD)[h]	28.6	28.0	0.60
B-Carotene-linoleic acid oxidation	AAC[i]	557	607	8.59
Sunflower oil-in-water emulsion oxidation	% Inhibition (peroxides)[j]	90.00	80.90	9.10
	Induction time (PV)[k]	35	31	12.12
	% Inhibition (CD)[l]	58.10	54.50	3.60
Methyl linoleate micelles oxidation	% Inhibition (TBARS)[m]	22.80	30.30	7.50
Rat liver microsomal peroxidation	% Inhibition (TBARS)[n]	51.91	41.07	10.84

Abbreviations: ABTS: 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant potential.[a] µmol Trolox equivalents/mg of dried extract; [b] µmol Trolox equivalents/mg of dried extract; [c] Scavenging (%) of $DPPH^{\bullet}$ (6×10^{-5} M) after 70 min; [d] Scavenging (%) of $DPPH^{\bullet}$ (3.04×10^{-5} M) after 20 min; [e] Effective concentration of dried extract (µg/ml) in reaction mixture required to scavenge 50% of $DPPH^{\bullet}$ (3.04×10^{-5} M); [f] $DPPH^{\bullet}$ rate of scavenging (s-1), calculated during unsteady state conditions (time 0 – 3 min), expressed as the change in the absorbance at 515 nm over time; [g] Concentration of dried extract (µg) per ml reaction mixture required to inhibit 50% of NBT reduction; [h] Inhibition (%) of conjugated diene (CD) formation after 21 h incubation at 40°C; [i] Antioxidant activity coefficient (AAC) measured as inhibition of β-carotene used); [j] Inhibition of peroxides after 35 days incubation at 30°C; [k] Time required for oxidation to reach a peroxide value (PV) of 10 meq/kg oil with incubation at 30°C; [l] Inhibition of CD formation after 31 days incubation at 30°C; [m] Inhibition of the formation of thiobarbituric reactive substances (TBARS) after 16 h incubation at 37°C; [n] Inhibition of the formation of TBARS during Fe^{2+}-induced microsomal lipid peroxidation after 1 h incubation at 37°C (Joubert et al., 2008a)

Liebert *et al*. (1999) found that TPC and antioxidant activity increased with brewing time, and that stirring while extracting tea resulted in black and green tea extracts with higher TPC and antioxidant activity than when prepared without stirring.

The position and degree of hydroxylation of the ring structure influences antioxidant activity (Krafczyk, Woyand, & Glomb, 2009). Free radicals are more effectively scavenged by an *ortho*-dihydroxyl functional group at the B-ring of flavonoids (Krafczyk *et al*., 2009). Flavonoids are oxidised to quinones after interception of radicals. A 3-hydroxyl functional group and 2,3-double bond conjugated to a 4-keto function at the flavonoid C-ring increases the antioxidant potential (Krafczyk *et al*., 2009). Dihydrochalcones are more active than their corresponding flavanones and flavones (Krafczyk *et al*., 2009). Krafczyk *et al*. (2009) found that isolated compounds from Rooibos had the following antioxidant activity in the TEAC assay (Trolox equivalents – TE), from highest to lowest: quercetin (2.70mM TE); dihydrocalcones aspalathin (2.62 mM TE) and nothofagin (2.06 mM TE); flavones orientin (1.47 mM TE), iso-orientin (1.54 mM TE), vitexin (0.86 mM TE), and iso-vitexin (0.81 mM TE); hyperoside (1.33 mM TE); iso-quercitrin (1.23 mM TE); flavonols rutin (1.20 mM TE); and flavanones (R)-eriodictyol-6-*C*-β-D-glucopyranoside (1.04 mM TE) and (S)-eriodictyol-6-*C*-β-D-glucopyranoside (0.88 mM TE).

According to Vinson *et al*. (1995), EGCG found in black tea is very effective in protecting against low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) oxidation and has a lipoprotein-bound antioxidant activity greater than tocopherol. A concentration dependent resistance to LDL oxidation was found for a black tea extract, which was equivalent to the effects of low concentrations of tocopherol (Nicolosi, Lawton & Wilson, 1999). ROS like superoxide radical ($O_2^{-\bullet}$), singlet oxygen ($^1O_2$), hydroxyl radical ($HO^\bullet$), peroxyl radical ($ROO^\bullet$), nitric oxide ($^\bullet NO$), nitrogen dioxide ($^\bullet NO_2$) and peroxynitrite ($O{=}NOO^-$), have been found to be trapped by tea preparations, resulting in reduced damage to lipid membranes, proteins, and nucleic acids in cell-free systems (Khan & Mukhtar, 2007). This is due to the vicinal dihydroxy or trihydroxy structure of catechins, that possess the ability to chelate metal ions and prevent the generation of free radicals, and also allows electron delocalization, imparting high reactivity to quench free radicals (Khan & Mukhtar, 2007). Catechins prevent free radical generation by inhibiting the activity of existing enzymes that generate these free radicals, such as xanthine oxidase, or by increasing the activity of enzymes with antioxidative properties by means of induction of protein molecule biosynthesis (Łuczaj & Skrzydlewska, 2005). Theaflavins are composed of more hydroxyl (OH) groups, which are needed for radical scavenging activity, and theaflavins also inhibit free radical generation and pro-oxidative enzymes activity (Łuczaj & Skrzydlewska, 2005). The strong interaction between polyphenols of black tea with transition

metals increases the antioxidative properties of black tea by forming complexes with iron and copper ions, inhibiting free radicals generation and the lipid peroxidation process (Łuczaj & Skrzydlewska, 2005).

Green tea has a higher concentration of catechin derivatives, such as (-)-EC, (-)-EGC and their gallates, ECG and EGCG and therefore is reported to have a higher antioxidant activity (Roy, Siddiqi, & Bhattacharya, 2001).

The antioxidant activity of green and black tea was directly compared to that of unfermented, semi-fermented and fermented black tea by von Gadow, Joubert and Hansmann (1997) (Table 2.5).

Table 2.5: Antioxidant activity of different teas as assessed with the DPPH radical scavenging and β-carotene bleaching methods

Type of tea	Inhibition (%) by DPPH assay	Antioxidant activity co-efficient by β-carotene bleaching method
Black	81.70[b]	650[ab]
Green	90.80[a]	695[a]
Unfermented Rooibos	86.60[b]	557[cd]
Semifermented Rooibos	81.90[b]	522[d]
Fermented Rooibos	83.40[b]	605[bc]

Means within a column followed by the same lowercase letter are not significantly different at P = 0.05 (von Gadow et al., 1997)

In this study it was found that the antioxidant activity of tea extracts tested using the DPPH radical scavenging method decreases in the order: green > unfermented Rooibos > fermented Rooibos > semifermented Rooibos > black (Table 2.5). Although levels were highest for green tea and statistically different from other tea types, all teas had high antioxidant activity. Using the β-carotene bleaching method, the antioxidant activity of tea extracts were found to be in decreasing order: green > black > fermented Rooibos > unfermented Rooibos > semifermented Rooibos (Table 2.5). Unfermented Rooibos (36.2%) had the highest total polyphenols (%), followed by fermented Rooibos (35.6%), green tea (34.9%) and black tea (33.9%). Green tea (33.0%) had the highest flavonoid content (%), followed by unfermented Rooibos tea (32.6%), black tea (32.0%) and fermented Rooibos tea (30.5%). Even though the water soluble solids of the different tea extracts contained roughly the same amount of total polyphenols and flavonoids, Rooibos contained less soluble solids than black tea (von Gadow *et al*., 1997). Therefore cup for

cup, fermented Rooibos tea would have a lower antioxidant activity then black tea (von Gadow *et al*., 1997).

Joubert, Winterton, Britz and Ferreira (2004) found that an aqueous extract of unfermented Rooibos tea (87.3%) contained a higher % Inhibition in the DPPH radical scavenging assay than an aqueous extract of fermented Rooibos tea (83.0%). These authors also determined the $DPPH^{\bullet}$ and superoxide anion ($O_2^{-\bullet}$) scavenging capacities of individual Rooibos flavonoids (Table 2.6).

Table 2.6: $DPPH^{\bullet}$ and $O_2^{-\bullet}$ scavenging capacities of Rooibos flavonoids compared to epicatechin, procyanidin B compounds, propyl gallate and Trolox

Compound	Inhibition of $DPPH^{\bullet}$ (%)[a]	Inhibition of $O_2^{-\bullet}$ (%)[b]
Rooibos flavonoids		
Quercetin	91.11	81.45
Orientin	88.65	72.52
Luteolin	88.01	57.83
Aspalathin	87.62	81.01
Isoquercitrin	86.59	66.67
Iso-orientin	82.18	63.32
(+)-Catechin	71.11	69.46
Rutin	66.75	68.16
Vitexin[c]	3.99	10.15
Chrysoeriol[c]	2.02	32.93
Controls		
Procyanidin B1	90.52	87.65
Procyanidin B2	91.19	91.87
Procyanidin B3	90.16	89.39
Procyanidin B4	90.16	89.64
Propyl gallate	86.11	93.90
(–)-Epicatechin	73.69	71.87
Trolox	58.10	5.31

[a] *All compounds, except vitexin and chrysoeriol, were tested at 0.25 mol/mol $DPPH^{\bullet}$.*
[b] *Compounds tested at 12.5 µmol/ml reaction medium.*
[c] *Tested at 0.5 ml/mol $DPPH^{\bullet}$.*
(Joubert et al., 2004)

Henning *et al*. (2003) determined the stability of flavonols responsible for the antioxidant activity of *Camellia sinensis* tea at the physiological pH. Most flavanols are unstable at pH 7, therefore results, especially those for the ORAC assay, may be underestimated. A buffer at pH 7 is used in the ORAC assay. Furthermore the ORAC assay takes 4 hrs during which time considerable degradation of flavonoids could occur. To determine their biological activity, the

stability of flavanols in different conditions such as pH and temperature must be taken into account. These authors found that at pH 7, catechin, epicatechin and ECG are quite stable, however EGC, EGCG, and GCG are completely degraded. These authors also reported that ORAC values ranged from 728 to 1372 µM TE/g tea for black tea and from 1239 to 1686 µM TE /g tea for green tea. The flavanol content is mainly responsible for the antioxidant capacity of tea. Other factors such as the thearubigin and rutin content could be the cause of the somewhat high ORAC value of some black teas with low flavanol and theaflavin content.

Bramati, Aquilano and Pietta (2003) found that hot aqueous extract of green tea (1,8 mg TE/g) had the highest total antioxidant activity measured by $ABTS^{\bullet+}$, followed by black tea (1,7 mg TE/g) and unfermented Rooibos tea (0,8 mg TE/g), with fermented Rooibos having the lowest antioxidant activity of 0,4 Trolox meq/g. The same trend was observed for TPC of the different types of tea, with green tea having 112,3 mg/g gallic acid equivalents (GAE), followed by black tea (105,0 mg/g GAE), and unfermented Rooibos tea (68,4 mg/g GAE). Fermented Rooibos tea had the lowest total phenolic content with 35,2 mg/g GAE.

A study done by Alarcón *et al*. (2008) reported that two black tea samples (677 and 553 mg GAE/L) had higher total phenolic content than green tea (517 mg GAE/L). In their study the antioxidant activity measured with the ORAC assay revealed higher values for the two black tea samples (2957 and 2329 mg GAE/L) than green tea (2086 mg GAE/L).

Venditti *et al*. (2010) found in their study that hot water steeping of tea resulted in higher TPC and antioxidant activity than cold water steeping. Green tea had a higher TPC and antioxidant activity (TEAC assay) than black tea, whether steeped in hot or cold water.

Green tea was found to have a much higher amount of polyphenols and flavanols and antioxidant activity as measured with the ORAC assay than unfermented and fermented Rooibos tea in a study done by Awoniyi *et al*. (2012) (Table 2.7). However, unfermented and fermented Rooibos had a higher flavonol content than green tea.

Table 2.7: Antioxidant profile of tea beverages

Treatment	Polyphenols (mg/L)	Flavanol (mg/L)	Flavonol (mg/L)	ORAC (mmol tea/L)
Rooibos fermented	981.16 ± 117.69	38.66 ± 8.06	299.33 ± 49.44	14556.81 ± 904.60
Rooibos unfermented	1354.33 ± 61.99	92.00 ± 2.60	247.00 ± 19.45	20888.75 ± 1281.03
Green tea	2723.16 ± 204.04	896.50 ± 25.44	108.66 ± 22.47	33350.68 ± 311.77

Aqueous solutions (2%) were prepared for fermented Rooibos, unfermented Rooibos, and green tea. ORAC, oxygen radical absorbance capacity (Awoniyi et al., 2012)

2.4 Nitric oxide scavenging ability

NO and reactive nitrogen species (RNS), which arise when NO and ROS interact, have been found to take part in the development of oxidative tissue/cellular damage (Tsai *et al*., 2007). Flavonoids act as antioxidants, and scavenge RNS and in doing so, may protect cells and plasma constituents against oxidative damage (Nijveldt *et al*., 2001; Coimbra *et al*., 2006).

Lin, Wu and Lin (2003) observed that green and black tea strongly inhibited the production of NO in cell culture. These authors found that (-)epigallocatechin-3-gallate in green tea and theaflavins in black tea were mostly responsible for suppressing NO production. Paquay *et al*. (2000) found that NO scavenging ability of green tea was five times stronger than that of black tea. Green tea, black tea and Rooibos tea had no significant effect on NO concentration *in vivo* (Persson *et al*., 2010). Sarkar and Bhaduri (2001) observed that black tea was more effective at inhibiting NO production than green tea, with theaflavin being the most powerful in inhibiting NO production. No literature could be found related to the NO scavenging activity of Rooibos tea.

2.5 Tea polyphenolics and protection against oxidative damage

The polyphenolics present in tea protect against oxidative stress which is the result of ROS and free radicals formation that causes extensive damage to DNA, proteins and lipids which leads to aging and degenerative diseases such as cancer, cardiovascular disease and diabetes (Limón-Pacheco & Gonsebatt, 2008). The ability of polyphenolics to reduce oxidative damage is based on the ability of polyphenolics to directly scavenge radicals, chelate metal ions thereby preventing the Fenton reaction, inhibiting and/or activating enzymes as well as being directly toxic towards cancer cells or indirectly through the formation of H_2O_2 which is cytotoxic.

ROS ($O_2^{-\bullet}$, $^1O_2$, $HO^\bullet$, $ROO^\bullet$, $^\bullet NO$, $^\bullet NO_2$, $O{=}NOO^-$) have been found to be trapped by tea preparations, resulting in reduced damage to lipid membranes, proteins, and nucleic acids in cell-free systems (Khan & Mukhtar, 2007). This is due to the vicinal dihydroxy or trihydroxy structure of catechins, that possess the ability to chelate metal ions and prevent the generation of free radicals, and also allows electron delocalization, imparting high reactivity to quench free radicals (Khan & Mukhtar, 2007). Catechins prevent free radical generation by inhibiting the activity of existing enzymes that generate these free radicals, such as xanthine oxidase, or by increasing antioxidant enzyme activity by increasing enzyme synthesis (Łuczaj & Skrzydlewska, 2005).

In cell culture media, tea polyphenols produce H_2O_2 (Feng *et al*., 2002). H_2O_2 produced by theaflavin was reported to induce apoptosis in several cancer cell lines as a result of a pro-oxidant effect of polyphenols (Feng *et al*., 2002). These authors also found that theaflavins and EGCG dispelled intracellular oxidative stress and protected cells, while at high concentrations these polyphenols were cytotoxic, with theaflavin being less cytotoxic than EGCG. These antioxidant, pro-oxidant effects as well as direct cytotoxicity is dependent on the metal-reducing potential, chelating behaviour, pH, solubility characteristics, the bioavailability, and stability in tissues and cells of polyphenols (Feng *et al*., 2002).

Babich, Gold and Gold (2005) tested the toxicity of green and black tea on human gingival epithelial-like (S-G) cells, and found that initial toxicity was noted at 100 µg/ml green tea polyphenol (GTP) extract and black tea polyphenol (BTP) extract, and toxicity increased as the concentration of GTP and BTP was progressively increased.

Theaflavins and thearubigens in black tea owe their antioxidant activity to their abilities to scavenge radicals and chelate metals, as well as their anti-mutagenic activities (Yang *et al*., 1998). EGC and theaflavins also have anti-proliferative or anti-carcinogenic activities. Yang *et al*. (1998) found that apoptosis and the growth inhibitory activity may be attributed in part to EGCG-induced production of H_2O_2 (Yang *et al*., 1998).

Cell apoptosis is induced due to the initiation of the cell redox system intracellularly to produce ROS (Yang *et al*., 2000). Yang *et al*. (2000) found that theaflavin digallate, EGCG, and EGC possesed strong growth inhibitory activity against human bronchial epithelial 33BES and 21BES

cell lines. Theaflavin gallate demonstrated a lower inhibitory activity and theaflavin was even less effective. Yang *et al*. (2000) suggested that the gallate structure of theaflavins is significant in growth inhibitory activity, and that the galloyl structure on the B ring of catechins is important in metal chelation, antioxidant activity and binding to cellular molecules.

Awoniyi *et al*. (2012) found that green tea had the highest protective effect in the 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) assay, with a relative fluorescence unit (RFU) of 3598.81 ± 990.76, followed by unfermented Rooibos tea (3061.82 ± 1021.66 RFU) and fermented Rooibos tea (2970.47 ± 352.13 RFU).

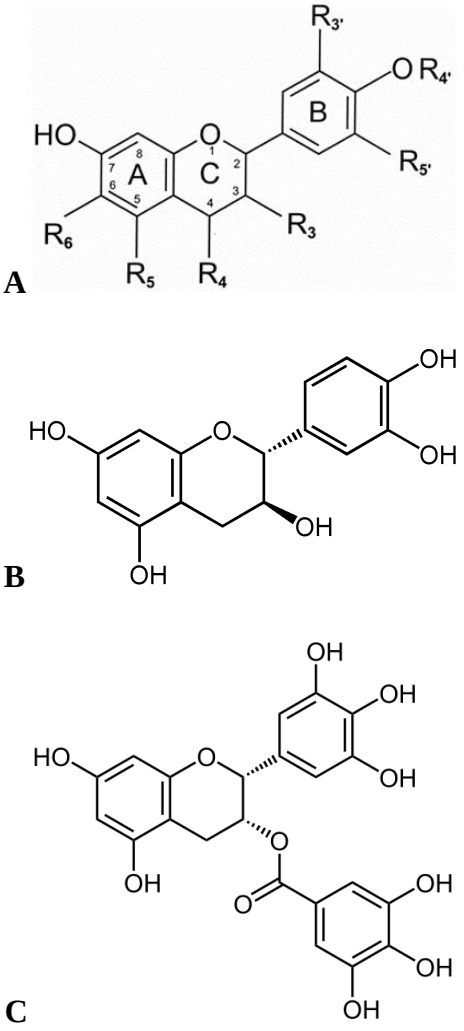

Figure 2.1: A) Basic structure of flavonoids (Patel, 2008), B) catechin and C) EGCG (Singh, Shankar & Srivastava, 2011)

According to Yoo *et al*. (2008), flavanols contain more hydroxyl groups, having 5 to 8 OH groups and these groups include *ortho*-(3′,4′) dihydroxy groups in the B-ring and 3-hydroxyl group and/or 3-galloyl group in the C-ring (Figure 2.1). The most potent antioxidant and radical scavenger among all of the polyphenols is EGCG which has 8 OH groups and *ortho*-(3′,4′)-dihydroxy groups in the B-ring as well as a 3-galloyl group in the C-ring. The flavan-3-ols are predominantly catechin derivatives that possess potent antioxidant activity. A study done by Yoo *et al*. (2008) found that ROS generation was the lowest for green tea, followed by black tea and then Rooibos tea, meaning that green tea had the highest protection, followed by black tea and then Rooibos tea. Green tea contained the most EGCG among 17 herb varieties and it contained almost twice as much total catechin as black tea. Catechin had a lower radical scavenging activity than other catechin derivatives, therefore the authors concluded it is possible that EGCG is the principal compound that contributes to the high antioxidant activity of green tea (Yoo *et al*., 2008).

In summary the effects in cellular systems is complex and involves chelation, direct scavenging, effects on protein expression, inhibition of radical producing enzymes, and is a function of concentration and structure of polyphenolics present in different tea types.

2.6 Effects of digestion on the antioxidant activity of tea

According to Ferruzzi (2010), *"Bioaccessibility... is defined as the fraction of phenolics transferred from the beverage matrix to continuous phase and available for uptake by the intestinal mucosa." In vivo*, there are some specific factors that may contribute to poor absorption efficiency of intact catechins from tea, and include the instability and solubility in the gut lumen, inefficient transepithelial transport, rapid phase II metabolism and clearance of catechins following absorption (Ferruzzi, 2010). Conditions present in the GIT lumen can affect the bioaccessibility of catechins, due to catechins being sensitive to near neutral (pH 6-7.5) conditions present in the small intestine, and as a result degrade in pH driven auto-oxidation type reactions. In *in vitro* digestion and cell culture media, a by-product of catechin auto-oxidation is known to be catechin auto-oxidation dimers that include theasinensins, which may have entirely different bioactivities than their native catechins and/or may change uptake and/or metabolism of monomeric catechins (Ferruzzi, 2010).

Record and Lane (2001) found that the green tea catechins GA, EGC, EGCG, EC and ECG levels were slightly lowered when incubated at pH 2 for 1 hour, while that of GCG increased slightly. In black tea, EGC, EGCG, GCG, and ECG were slightly lowered when incubated at pH 2 for 1 hour. All green and black tea catechins were drastically reduced when incubated at pH 7 for 15 to 60 minutes. This showed that various catechins, especially EGCG and EGC, were unstable and degraded under neutral/alkaline conditions (Record & Lane, 2001). The oxidation reaction can occur in foods and beverages that contain phenolic compounds (Cilliers & Singleton, 1989; Spencer, 2003). This process is nomally slow under acidic conditions, however at higher pH oxidation occurs quite rapidly. Neutral and alkaline pH environments of the small intestine and colon cause polyphenolic compounds to oxidise. Oxidised products sometimes possess greater radical scavenging activity than their original flavonoids (Spencer, 2003).

Studies have shown that catechins are stable in acid but are severely degraded in fluids of near neutral or greater pH, such as intestinal juice, plasma, bile, cell culture media, or simulated digestive conditions (Neilson *et al*., 2007). At higher pH, EGCG degrades by epimerization and autoxidation reactions involving the B-ring, causing the formation of homocatechin dimers such as theasinensin (THSN) A, THSN D, and P-2 (Figure 2.2). These are minor components of mildly fermented tea such as oolong and black tea. Catechins with similar structures may also undergo dimerization, with the subsequent formation of products analogous to theasinensins and P-2. Higher pH (6-7.5), digestive secretions (bile, etc.), and dissolved O_2 and ROS are all conditions present in the upper small intestine that favour autoxidation during human digestion. Thus the possibility exists for preabsorptive catechin autoxidation in the gut lumen.

Using HPLC-DAD, Neilson *et al*. (2007) tested the digestive losses of individual catechins. Significant losses of 71-91, 72-100, and 60-61% were observed for EGCG, EGC, and ECG, respectively. EC and C on the other hand, were not as much affected by digestion, with losses of 8-11 and 7-8%, respectively. Catechins (C and EC) are more stable than gallocatechins (EGC and EGCG) when exposed to digestive conditions, while catechin gallates (ECG) have intermediate stability. Intestinal phase degradation was more profound than gastric phase losses. Gastric degradation of 5-7, 3-12, and 5-6% occurred for EGCG, EGC and ECG, respectively. Gastric phase losses for C and EC were 1-2 and 0.1-0.2%, respectively. Intestinal losses were 65-86, 67-88, and 52-55% for EGCG, EGC and ECG, respectively. C and EC degraded with 7-8 and

7-10% during intestinal digestion, respectively. Therefore, intestinal phase losses were 10-20 fold higher than that for gastric phase losses, proposing that catechins are relatively more stable under gastric phase conditions than under intestinal phase conditions of digestion. From a nutritional perspective, the loss of polyphenolic compounds observed during gastric digestion is insignificant. However, degradation during intestinal digestion drastically decreased the nutritional antioxidant content of the tea. Neilson *et al*. (2007) also observed that degradation of catechins under digestive conditions seem to be more directly related to pH than to digestive enzyme activity.

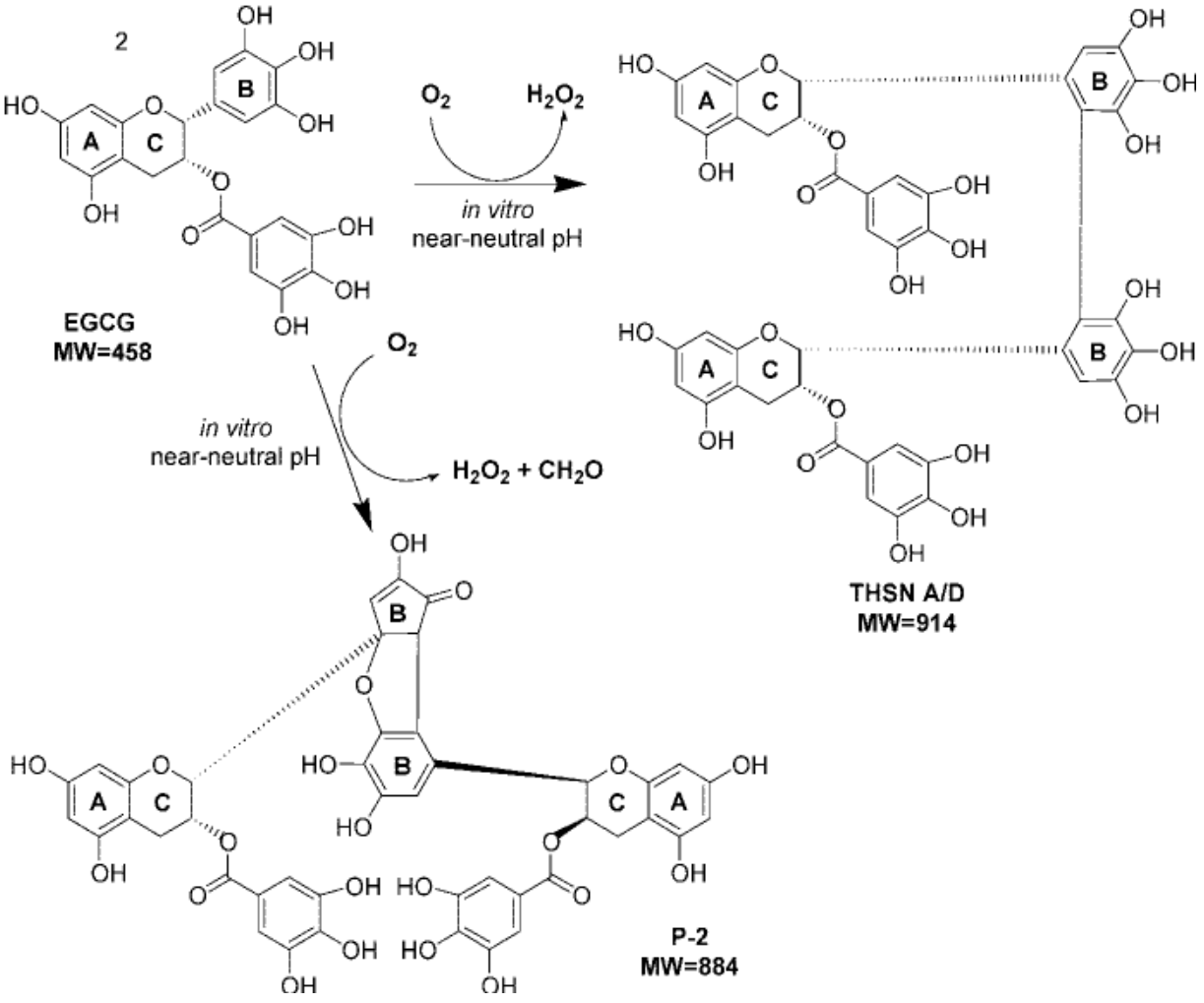

Figure 2.2: Autoxidation reactions of EGCG at near-neutral pH. Two EGCG monomers form a C – C bond in the B-ring, resulting in the net loss of two hydrogen atoms, to generate the homodimers theasinensin (THSN) A and THSN D. Two EGCG monomers also undergo B-ring opening and subsequent condensation, resulting in the net loss of two hydrogen atoms and formaldehyde (CH_2O), to generate the homodimer P-2 (Neilson *et al*., 2007)

Under *in vitro* digestive conditions, dimers that are catechin autoxidative products were detected (Neilson *et al*., 2007). These products are formed at near-neutral or greater pH when catechins scavenge O_2 to produce superoxide, producing a semiquinone intermediate (Figure 2.2). O_2 or superoxide is then scavenged by a second catechin monomer, resulting in the dimerization of the two semiquinone intermediates.

Green tea catechins were stable in pH conditions lower than pH 4, while above pH 8 these catechins were extremely unstable and degraded almost completely (Zhu *et al*., 1997). Between pH 4-8, the stability of catechins were pH dependent, i.e. the lower the pH the greater the stability. EGCG and EGC are particularly instable in neutral or alkaline pH conditions. Su *et al*. (2003) observed that catechins and theaflavins are relatively stable at pH 4, however some degradation does occur, and in contrast very unstable under neutral conditions. The same results were found by Zhu *et al*. (1997) regarding green tea catechins.

Phenolic compounds, such as flavonoids, exist in plants as structures known as glycosides and aglycones. Acidic or alkaline hydrolysis breaks the glycoside linkages (Cai *et al*., 2004; Khoddami, Wilkes & Roberts, 2013), resulting in the release of flavonoids.

After simulated gastric and small intestinal digestion of green tea, Green *et al*. (2007) found that the overall catechin digestive recovery from green tea preparations was poor, with roughly 80.4 ± 1.46% loss of total catechins. These authors observed that catechin degradation during though the gastic portion of the *in vitro* digestion model was not significant, and that primary degradation of catechins occurred during the small intestinal phase. EGC and EGCG were most prone to degradation, whereas EC and ECG were relatively stable. EGC and EGCG accounted for more that 80% of the total catechin content of green tea used in their study. Elevated pH (6.0-8.0), residual dissolved oxygen, and the expected occurrence of ROS from normal digestive function could facilitate many reactions including epimerization and auto-oxidation in the intestinal lumen (Green *et al*., 2007).

Yoshino *et al*. (1999) found that P-1 and P-2, two dimerization products formed easily from EGCG in rat plasma and bile, i.e. mildly alkaline fluids, were increased during simulated digestion. These authors did not include gastric enzymes in their study and neglected the effects of proteins on the degradation of catechins. The antioxidant values of the dimerization products

from EGCG in mild alkaline fluids were compared to that of EGCG. Fe^{2+}-chelating activities of the products were almost two times that of EGCG. O_2-Scavenging activities of P-2 were considerably higher than those of EGCG.

The antioxidant activity as measured by Krul *et al*. (2001) of green tea and black tea in their study was 24.4 ± 4.4 and 15.4 ± 1.6 mM TE/L, respectively. These were dialysates of the jejunum taken from their *in vitro* digestion model. The amount of catechins (mg) measured in the jejunal dialysates of this model is presented below (Table 2.8).

Table 2.8: Catechin content (mg) of jejunal dialysates of green tea (10 g) and black tea (10 g)

Tea	GC	EGC	C	EGCG	EC	GCG	ECG	Sum of Catechins
Green	49.2±9.8	262.7±70.0	68.1±6.9	151.4±30.2	172.2±17.4	21.5±1.9	84.8±28.8	**809.9±161.0**
Black	6.7±2.9	19.7±5.3	9.8±0.4	19.2±6.3	29.3±1.6	29.1±1.6	13.4±3.9	**127.0±21.8**

(Krul et al., 2001)

Krul *et al*. (2001) found that EGC was the most abundant catechin in the jejunal dialysates, followed by EC and EGCG. Catechin gallate was not detected in any of the dialysates. The major components of green tea are catechins, with about 90% of dry weight. On the other hand, black tea is composed of less catechins but higher proportions of other compounds such as theaflavins (10%), thearubigins (50%), and gallic acids (hydrolyzable tannins). These compounds, formed during fermentation of black tea, also have antioxidant activity.

According to Hollman, Tijburg and Yang (1997), polyphenols have a strong affinity for proline-rich proteins. The binding capacity of large, flexible, poorly water soluble polyphenols are the greatest. Digestive enzymes can also be bound to tannins (Bravo, 1998), specifically condensed tannins. This ability is however limited to those molecules that physically have access to soluble proteins. Tannins also have the ability to bind to endogenous proteins in the GIT, for instance, digestive enzymes, and thereby inhibiting the activity of these enzymes (Bravo, 1998; Bandyopadhyay, Ghosh & Ghosh, 2012). In contrast, Cilla *et al*. (2011) stated that digestive enzymes release bound antioxidants from the food matrix, resulting in these compounds becoming available. The molecular structure of tea polyphenols is composed of hydroxyl and galloyl groups, and can therefore form hydrogen bonds with polar groups (amide, guanidine, peptide, amino and carboxyl groups) of proteins, as well as hydrophobic association between galloyl groups and hydrophobic amino acids in enzyme protein (proline, phenylalanine and

tyrosine) (He, Lv & Yao, 2006). Hydrogen bonding and hydrophobic association will manipulate the configuration of enzymes, thereby changing their activity.

Viljoen (2008, pp. 177-178) found that with increasing pH and temperature, the aspalathin content of fermented Rooibos tea decreased. Significant ($p < 0.05$) reductions in aspalathin content of unfermented Rooibos tea was seen at pH 6 (30 and 40°C) and pH 7 (all temperatures), however no significant ($p \geq 0.05$) change was seen at pH 3, 4 or 5. Fermented Rooibos tea's iso-orientin content was was not as greatly influenced by changes in pH and temperature as its aspalathin content. Regardless of storage temperatures, no significant ($p \geq 0.05$) reduction in iso-orientin was seen for fermented Rooibos at pH 3, 4 or 5. A significant ($p < 0.05$) decrease in iso-orientin content was observed at pH 6 (40°C). These decreases were more pronounced at pH 7 (40°C), but iso-orientin content of the sample at 30°C was also significantly ($p < 0.05$) decreased. At pH 3 and 4, iso-orientin content of unfermented Rooibos was unaffected, however at pH 5 (40°C) iso-orientin content was significantly ($p < 0.05$) higher. Significant decreases ($p < 0.05$) in iso-orientin content was observed for all samples stored at pH 6 (40°C). At pH 7 (30 and 40°C), these reductions were even more profound. As with iso-orientin, the orientin content of fermented Rooibos tea was not as greatly affected by changes in pH and temperature as aspalathin content was. Between pH 3 and 5, no significant ($p \geq 0.05$) change in orientin content of fermented Rooibos was seen. A significant ($p < 0.05$) reduction in orientin content was observed at pH 6 (30°C) and pH 7 (30 and 40°C). The orientin content of unfermented Rooibos was mostly unaffected at all pH values. Significant ($p < 0.05$) increases of 4.9 and 3.2% in orientin content was seen at pH 5 and 7 (40°C).

Nanjo *et al*. (1996) found that scavenging abilities (+)-C and (-)-EC decreased significantly from pH 7 to 4, with their effects at pH 4 being 10 times weaker than at pH 7. Scavenging ability of (-)-EGC was higher at pH 7 than at pH 4. Nanjo *et al*. (1996) suggested that the *ortho*trihydroxyl group in the B-ring and the galloyl moiety added to retaining the DPPH radical scavenging ability efficiently at lower pH. These authors also stated that the scavenging ability of the ortho-dihydroxyl group in the B-ring is limited in neutral and alkaline conditions. Nanjo *et al*. (1996) concluded that (-)-EGC, (-)-ECG, and (-)-EGCG functional scavengers over a wide range of pHs, while (+)-C and (-)-EC are limited in neutral and alkaline pH conditions. Wootton-Beard, Moran and Ryan (2011) observed that after stomach digestion, radical scavenging ability of juice

increased slightly, while after duodenal digestion it decreased slightly. A similar trend was seen with tea juices, which increased in DPPH scavenging ability after stomach digestion, and then decreased significantly after duodenal digestion (Chen *et al*., 2013).

Antioxidant activity as measured by the TEAC assay increased significantly after *in vitro* stomach digestion and retained this increase after duodenal digestion (Wootton-Beard *et al*., 2011). Chen *et al*. (2013) observed that tea juices decreased significantly in antioxidant activity in the TEAC assay after *in vitro* stomach digestion, which was followed by further decrease after duodenal digestion.

To summarise, in the case of green, black and Rooibos tea, antioxidant activity is either stable or slightly lowed during stomach digestion or similar conditions, whereas at neutral conditions present during intestinal digestion, significant decreases are observed in antioxidant properties. Numerous factors affect tea polyphenols during digestion, such as pH conditions, instability and solubility in the GIT lumen, auto-oxidation type reactions, proteins and digestive enzymes.

2.7 Conclusion

This literature reviewed has highlighted limitations in our knowledge regarding the effect of digestion on the antioxidant content and activity of fermented and unfermented Rooibos tea. In addition the effect of digestion on NO scavenging of all types of tea is unknown. No literature was found about the effect of pH or digestion on the antioxidant activity of tea as measured by the DCFH-DA assay in cellular models, which further raises the question, do changes in antioxidant content and activity of tea also result in significant changes in cellular protective effects? Both *Camellia sinensis* and *Aspalathus linearis* tea are consumed by adults, children and infants and little is known about the effect of infant digestion on all measured parameters. Lastly the question is raised whether *Camellia sinensis* and *Aspalathus linearis* tea as consumed in South Africa contribute significantly to the antioxidant status of this population. Therefore this study was undertaken to determine what the effect of adult and infant stomach and duodenal digestion of fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea has on their respective antioxidant content and activity.

Chapter 3: Antioxidant properties of commercially available South African teas *Camellia sinensis* and *Aspalathus linearis*

3.1 Abstract

Polyphenolic antioxidants, particularly flavonoids, in tea offer protection by suppressing ROS, scavenging ROS, and protecting antioxidant systems. Oxidative stress caused by ROS and free radicals leads to damage of DNA, proteins and lipids, contributing to degenerative diseases such as cancer, cardiovascular disease and diabetes. The antioxidant content and activity of commercially available *Camellia sinensis* and *Aspalathus linearis* tea were determined. This included the ability to protect against oxidative damage in a cellular environment. Total polyphenolic (TPC) and flavonoid (TFC) content was determined by Folin-Ciocalteu and aluminium chloride assays, respectively, whereas antioxidant activity was determined by DPPH radical scavenging, TEAC and ORAC assays. Oxidative damage was determined by NO scavenging assay and Caco-2 cell models. Black tea had the highest antioxidant content (28.23 mg GAE/g; 89.87 mg CE/g), followed by green tea (27.86 mg GAE/g; 71.67 mg CE/g), unfermented Rooibos tea (12.09 mg GAE/g; 68.56 mg CE/g), and fermented Rooibos tea (8.81 mg GAE/g; 59.30 mg CE/g). The same trend was observed in the TEAC and ORAC assays, whereas in the DPPH assay green tea had the highest antioxidant activity (129.88 mg TE/g), followed by black tea (125.68 mg TE/g), unfermented Rooibos tea (64.30 mg TE/g), and fermented Rooibos tea (46.20 mg TE/g). Both teas effectively scavenged NO and this effect was 1.96 fold greater for fermented black tea. On a cellular level, teas offered 84% or more protection against oxidative damage. Finally the calculated RSC was that one cup of black tea and two cups of Rooibos tea would be sufficient to meet 200 mg vitamin C equivalent antioxidant intake.

3.2 Introduction

Tea is the most commonly consumed beverage in the world, next to water, with an estimated 18-20 billion cups being consumed daily (Marcos *et al*., 1998). Black tea is commonly consumed in the U.S., Europe, Africa and India (Wiseman *et al*., 1997), whereas green tea is mostly consumed in Asia, particularly in Japan and China, North Africa and the Middle East (Balentine *et al*.,

1997). In South Africa, tea is drunk by all sectors of the population, with black tea typically consumed in the North-West province (Breet *et al*., 2005), and Rooibos tea in the Western Cape (Faber & Benadé, 2007). In the rest of South Africa tea consumption is usually based on personal choice i.e. factors such as tradition, taste, cost and reported health benefits. Tea can be classified as unfermented, semi-fermented, and fermented. When tea leaves are unwilted and unoxidised, it is unfermented tea, and when tea leaves are wilted and fully oxidised, it is fermented tea. *Camellia sinensis* green tea and *Aspalathus linearis* green Rooibos tea are examples of unfermented tea (Ferruzzi, 2010). *Camellia sinensis* black tea and *Aspalathus linearis* Rooibos tea are examples of fermented tea (Ferruzzi, 2010).

Oxidative stress is a common feature of cancer, cardiovascular disease, diabetes, neurodegenerative disorders and HIV (Moskaug *et al*., 2005). Tea polyphenols scavenge free radicals and are potent antioxidants and it is hypothesised that can inhibit or reduce the effects of oxidative stress (Pietta, 2000). Research has also found that green tea catechin EGCG inhibits HIV-1 infectivity by preventing the binding of HIV-1-glycoprotein 120 to the CD4 molecule (Nance, Siwak & Shearer, 2009). Therefore tea and other polyphenol rich products can be a source of antioxidant molecules that can reduce oxidative damage as well as a source of novel therapeutic molecules.

The polyphenols present in black tea (*Camellia sinensis*) are the catechins, EGCG, EGC, ECG, and EC (Łuczaj & Skrzydlewska, 2005) while gallocatechin, epigallocatechin digallate, 3-methylepicatechin gallate, catechin gallate, and gallocatechin gallate are catechins that are present in smaller amounts (Łuczaj & Skrzydlewska, 2005).

Vinson *et al*. (1995) observed that oxidation of LDL and VLDL *in vitro* is reduced by EGCG in black tea, which has a lipoprotein-bound antioxidant activity greater than tocopherol. In an animal study done by Nicolosi *et al*. (1999), black tea extract increased the resistance of LDL to oxidation in a concentration dependent manner, although tocopherol was more effective at low concentrations. Khan and Mukhtar (2007) found that damage caused by ROS ($O_2^{-\bullet}$, $^1O_2$, $HO^\bullet$, $ROO^\bullet$, $^\bullet NO$, $^\bullet NO_2$, $O{=}NOO^-$) to lipid membranes, proteins, and nucleic acids in cell-free systems is reduced by tea. The vicinal dihydroxy or trihydroxy structure of catechins in tea possess the ability to chelate metal ions and prevent the generation of free radicals, and also allows electron delocalization, imparting high reactivity to quench free radicals (Khan & Mukhtar, 2007). Free-

radical generation is prevented by catechins, which inhibit the activity of free-radical generating enzymes such as xanthine oxidase, or by increasing the activity of enzymes with antioxidative properties by means of induction of protein biosynthesis (Łuczaj & Skrzydlewska, 2005).

Black tea also contains theaflavins that also inhibit free radical generation and pro-oxidative enzymes activity (Łuczaj & Skrzydlewska, 2005). Black tea polyphenols and transition metals form strong interactions, increasing the antioxidant property of black tea by forming complexes with iron and copper ions, thereby inhibiting the generation of free radicals and the process of lipid peroxidation (Łuczaj & Skrzydlewska, 2005).

Whereas black tea contains caffeine, Rooibos (*Aspalathus linearis*) is naturally caffeine free, and is also a low tannin beverage when compared to *Camellia sinensis* teas (Joubert *et al*., 2008a). Aspalathin, a C – C linked dihydrochalcone glucoside, is a unique monomeric flavonoid compound found in Rooibos tea (Koeppen & Roux, 1965a; Koeppen & Roux, 1966; Rabe *et al*., 1994), and the cyclic dihydrochalcone aspalanin is currently known to only be isolated from *Aspalathus linearis* (Shimamura *et al*., 2006). Nothofagin, a rare 3-dehydroxy dihydrochalcone glucoside compound in Rooibos, is also present in the heartwood of *Nothofagus fusca* (Hillis & Inoue, 1967). Other C – C linked β-D-glucopyranosides found in Rooibos include the flavones orientin, iso-orientin (Koeppen & Roux, 1965b), vitexin and isovitexin (Rabe *et al*., 1994), and the flavanones dihydro-orientin, dihydro-iso-orientin (Bramati *et al*., 2002) and hemiphlorin (Shimamura *et al*., 2006).

As a result of the oxidation processes of fermented Rooibos tea, lower amounts of flavonoids, particularly aspalathin, are found, resulting in fermented Rooibos tea's antioxidant capacity being lower than that of unfermented Rooibos tea (Standley *et al*., 2001). Using Chinese hamster lung fibroblast V79-4 cells, Yoo *et al*. (2008) found that Rooibos had a protective effect against oxidative stress caused by H_2O_2, and that the antioxidant enzymes superoxide dismutase and catalase were induced in the V79-4 cells by Rooibos. Studies have shown that Rooibos protects against lipid peroxidation in linoleic acid emulsions (Joubert *et al*., 2005) and also protects rat liver microsomes (Joubert *et al*., 2008b). As TPC is reduced with fermentation, the antioxidant activity, in terms of $O_2^{\bullet -}$, $DPPH^{\bullet}$, $ABTS^{\bullet +}$ and FRAP, also decreases from unfermented to fermented Rooibos hot water extracts (Joubert *et al*., 2008a).

Even though water soluble solids of the different tea extracts contain roughly the same amount of total polyphenols and flavonoids, Rooibos tea contains less soluble solids than black tea (von Gadow *et al*., 1997). Therefore cup for cup, fermented Rooibos tea would have a lower antioxidant activity than black tea (von Gadow *et al*., 1997).

The purpose of this study was to determine the antioxidant content and activity, NO scavenging as well as the cellular protective effects of locally available unfermented and fermented *Camellia sinensis* and *Aspalathus linearis* teas. Then to determine whether the relative scavenging content (RSC) of a 250 ml cup of tea will contribute significantly to daily dietary antioxidant requirements.

3.3 Materials and Methods

3.3.1 Sample preparation

Tea samples were obtained during June 2011 from various retail outlets. Four black tea samples were used (Woolworths brand – W; Pick 'n Pay brand – P; Five Roses – F; Glen – G), three fermented Rooibos samples (Laager – L; Freshpak – F; Pick 'n Pay brand – P), one green tea sample (Woolworths brand – W) and unfermented Rooibos (UR) (Laager – L) sample were obtained. Samples were stored in the dark at room temperature. The mass of tea in each teabag was determined, as usually tea is prepared by steeping a single tea bag in 200 ml water. Two grams of unfermented and fermented Rooibos (*Aspalathus linearis*) and black and green (*Camellia sinensis*) tea from tea bags were steeped for 3 min in 200 ml distilled water under controlled laboratory conditions. For total polyphenolic content, total flavonoid content and DPPH radical scavenging assays a 25% black tea and green tea as well as 50% fermented and unfermented Rooibos tea solutions were prepared. For the TEAC, ORAC, NO and DCFH-DA assays a further 10 time dilution of the stock solutions were prepared. These stock solutions were stored at -20°C in the dark until further analysis. Although not widely consumed in South Africa, green tea (*Camellia sinensis*) and unfermented Rooibos tea (*Aspalathus linearis*) were used as unfermented tea controls for black tea and fermented Rooibos tea, respectively.

3.3.2 Total polyphenol content (TPC)

Total polyphenolic content (TPC) was measured by the Folin-Ciocalteu (F-C) assay, which is based on a reduction-oxidation reaction involving oxidation of the phenolate ions under alkaline

conditions while reducing the phosphotungstic-phospho-molybdic complex in the reagent to a blue coloured solution (Waterman & Mole, 1994). The F-C method described by Serem and Bester (2012) was used to determine TPC, modified for a 96 well format. The standard curve was prepared using gallic acid (0-0.03 mg/ml). Tea solution of 10 µl volume was added to the microplate, followed by 50 µl volume of F-C, and then 50 µl volume of a 7.5% sodium carbonate solution. The mixture was mixed well and the absorbance was read at 630 nm. For each sample a blank consisting of 10 µl of a tea solution with 100 µl PBS was run to correct for any colour interference. TPC was expressed as mg/g gallic acid equivalents (GAE).

3.3.3 Total flavonoid content (TFC)

Total flavonoid content (TFC) was evaluated using the aluminium chloride assay, which is based on the formation of a red aluminium complex where the flavonoid acts as a bidental ligand, forming complexes with the C-4 keto group and either the C-3 or C-5 OH group of flavones and flavonols (Amaral *et al.*, 2009). A modified method of Serem and Bester (2012) was used to measure TFC. The standard curve was prepared using catechin (0-0.21 mg/ml). Tea solution of 10 µl volume was added to each well of a 96 well microplate. To each well, 30 µl of a 2.5% sodium nitrite, followed by a 20 µl of a 2.5% aluminum chloride and then 100 µl of a 2% sodium hydroxide were then added. After mixing properly the absorbance was read at 450 nm using a BioTek plate reader. As for the determination of TPC a 10 µl of a tea solution with 150 µl PBS was used as a blank. TFC of each tea sample was expressed as mg/g catechin equivalents (CE).

3.3.4 DPPH radical scavenging assay

DPPH radical scavenging ability was measured using the method as described by Serem and Bester (2012). Preparation of DPPH stock solutions was done by dissolving 24 mg of DPPH in 100 ml methanol, which was then shaken in a sonicator for 20 min. Working solutions were prepared by diluting 20 ml stock solution with 80 ml methanol. The standard curve was made using Trolox (25 mg/ml), which was linear between 0 and 800 µM. To 15 µl of tea solution, 285 µl of DPPH was added. The microplate was then left to stand for 15 min in a dark place. Each sample acted as its own control to eliminate possible effects of interference, i.e. all components, no DPPH added. The plate was then read at 570 nm and the data was expressed as mg/g Trolox equivalents (TE).

3.3.5 TEAC assay

$ABTS^{\bullet+}$ was freshly produced by adding 3 mM of potassium peroxodisulphate ($K_2S_2O_8$) solution to 8 mM ABTS and the mixture was left to react in the dark for at least 12 h at room temperature. ABTS stock solution was diluted with 0.2 M (pH 7.4) phosphate buffer (PBS) to prepare the working solution. The standard used was Trolox, concentration range 0-1000 µM. To 0.1 ml of tea solution was added 2.9 ml volume of working solution. The reaction mixtures were left to stand at room temperature and the absorbance readings were taken at 630 nm after 15 min for the standards and 30 min for the samples, using the BioTek spectrophotometer. Tea samples were left for a longer reaction time, as the standards were simple solutions and tea solution were complex food matrices. Each sample acted as its own control i.e. all components, no ABTS added. Data was expressed as mg/g Trolox equivalents (TE).

3.3.6 ORAC assay

In the ORAC assay the peroxyl radical generator was AAPH, the standard was Trolox (0 – 1000 µM) and the fluorescent probe used was fluorescein. PBS was used as a blank. A stock solution was prepared by mixing 3.76 mg of fluorescein with 50 ml PBS buffer. The working solution was prepared by mixing 140 µl of the stock solution with 5 ml PBS buffer and 45 ml distilled water. To 0.08g AAPH in 4 ml distilled water was then added 160 µl of the above working solution (Fluorescein-AAPH mixture). After adding 10 µl Trolox standards and tea solutions into separate wells in a 96 well microplate, 200 µl of the fluorescein-AAPH mixture was then added to the wells containing the standards and tea solutions. Samples were mixed and the microplate placed in the plate reader and incubated at 37°C (Serem and Bester (2012)). The fluorescence was measured every 5 min for 4h. The assay protocol included: measurement start time of 0.0s, 10 flashes per cycle, 300 s cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The final ORAC values of the samples were calculated by using the net area under the decay curves (AUC) and the data was expressed as mg/g Trolox equivalents (TE).

3.3.7 Nitric oxide scavenging assay

Sodium nitroprusside at physiological pH spontaneously releases NO which can be used to evaluate the NO scavenging capacity of compounds. Sodium nitroprusside (0-0.1 mM) was used as the standard. Eighty microlitres of 5 mM sodium nitroprusside was added to 20 µl of tea (10% v/v) sample and trolox standards (10% v/v) and incubated for 1 h in the dark at room

temperature. Fifty µl of a 0.33% sulphanilamide solution (w/v) was then added to each well containing tea or trolox and the solution was incubated for a further 10 min in the dark. Then 50 µl of a 0.1% NED (N-1-naphthylethylenediamine dihydrochloride) solution was added before the absorbance was read at 550 nm.

3.3.8 Cellular oxidative damage

Figure 3.1 shows how cytotoxicity due to oxidative damage was determined. A 20 µM solution of DCFH-DA was prepared which was a 1 mg/10 ml, 200 µM stock solution, from which a 20 µM working solution was prepared. To each well was added 40 µl of the DCFH-DA solution. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Every 2 min, the change in fluorescence from 0-60 min was measured using a FLUOstar OPTIMA plate reader.

3.3.9 Total cellular protective effects

Total protective effects were evaluated as shown in Figure 3.1. For this 20 µM solution of DCFH-DA was prepared as described above. DCFH-DA solution of 40 µl was added to each well and cell culture plates were incubated for 1 h at 37°C. The medium containing the DCFH-DA solution was then carefully removed. Using a plastic pipette, PBS was used to wash each well in the cell culture plate once, which was then removed and the plates blotted dry. A volume of 40 µl tea sample was then added to each well of the cell culture plates followed by a 15 mM, 40 µl volume of AAPH, the AAPH having a final concentration of 7.5 mM, and the tea samples having a final concentration of 0.01 and 10% (w/v). Change in fluorescence was measured immediately over 0-60 min, every 2 min. The gradient of the change in fluorescence was calculated, and the data was expressed as % damage where AAPH alone causes 100% damage.

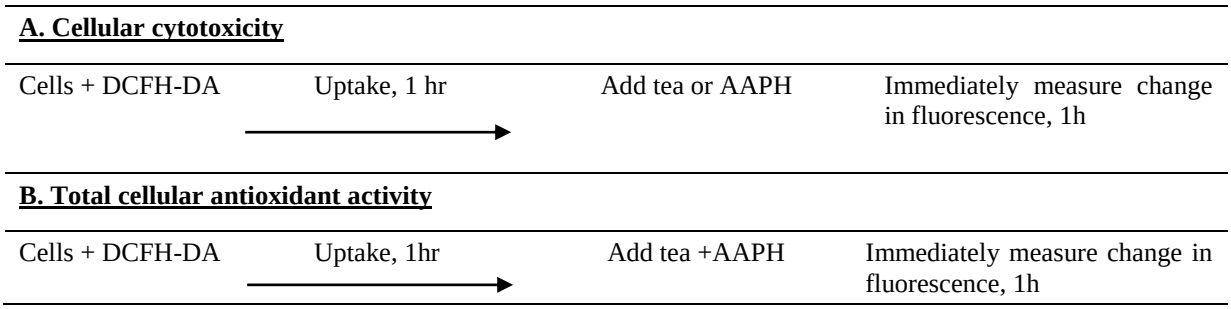

Figure 3.1: Schematic diagram of the strategy used to determine the cytotoxicity and total antioxidant activity

3.3.10 Data management and statistical analysis

The means were subject to one-way ANOVA, calculated at the 5% level using STATISTICA Version 8 to compare treatment means for TPC, TFC, the antioxidant activity assays (DPPH, TEAC and ORAC assays), NO scavenging and DCHF-DA assays. One-way ANOVA was used when more than two sample means were compared.

Statistical significance between data does not necessary indicate biological relevance and for this reason a difference of ≥ 10% was considered to be biologically relevant. The European Food Safety Authority (EFSA) Scientific Committee (2011) stated that *"Statistical significance is considered as just one part of an appropriate statistical analysis of a well designed experiment or study. Identifying statistical significance should not be the primary objective of a statistical analysis". "A biologically relevant effect can be defined as an effect considered by expert judgement as important and meaningful for human, animal, plant or environmental health. It therefore implies a change that may alter how decisions for a specific problem are taken"* (EFSA Scientific Committee, 2011). According to Lovell (2013), the judgement in the choice for the biological relevance effect size is a subjective decision based on the scientist's knowledge.

3.4 Results and Discussion

To evaluate the health benefits of *Camellia sinensis* and *Aspalathus linearis* tea as consumed by the South African population and standardize all findings as mg/g dry tea material, the average mass of the content of each teabag was determined (Table 3.1). The average mass of the content of teabags were 2.32 ± 0.26 g and 2.51 ± 0.13 g for black tea and fermented Rooibos tea, respectively. All data was expressed as mg or µmol per dry mass (g) tea. To determine the physiological relevance of measured content and activity, in the summaries the data was expressed as mg per mL tea as this reflects the activity of tea as it is consumed (Table 3.4).

3.4.1 Antioxidant content: TPC and TFC

Per gram black tea had the highest TPC (26.85-33.00 mg GAE/g), followed by green tea (27.86 mg GAE/g), then unfermented Rooibos tea (12.09 mg GAE/g), and fermented Rooibos tea (7.62-9.55 mg GAE/g) (Table 3.2). Black tea (75.05-103.66 mg CE/g) and green tea (71.67 mg CE/g) also had the highest TFC, followed by unfermented Rooibos tea (68.56 mg CE/g), and with fermented Rooibos tea having the lowest TFC (49.64-69.57 mg CE/g). For the principal tea types used in this study the TPC of black tea was 2.52- 3.46 fold greater than fermented Rooibos tea while the TFC of black tea was 1.48-1.51 fold greater than Rooibos tea. This data differs from the data presented in Table 2.7. In the study of Awoniyi *et al*. (2012), a 2g/100ml extract was used and antioxidant content and activity was determined after 30 minutes, therefore measured activity would be considerably greater than found in this study.

Table 3.1: Mass of tea content of teabags of *Camellia sinensis* and *Aspalathus linearis* teas

Sample	Mass (g)
Fermented	
Black tea	
W	1.93 ± 0.07
G	2.40 ± 0.11
F	2.47 ± 0.03
P	2.47 ± 0.07
Average	**2.32 ± 0.26**
Rooibos tea	
F	2.59 ± 0.08
L	2.58 ± 0.06
P	2.35 ± 0.09
Average	**2.51 ± 0.13**
Unfermented-Controls	
Green tea – W	1.95 ± 0.04
Rooibos tea – L	2.52 ± 0.05

Data is an average of 3 experiments ± SD.

The difference in TPC and TFC of unfermented and fermented tea is due to oxidative changes that occur during the fermentation process (Pellegrini *et al*., 2003; Standley *et al*., 2001). Flavanols in green tea leaves, specifically catechins and their associated gallic acid residues, are converted by polyphenol oxidase in an oxidative polymerization reaction to thearubigins and theaflavins which turns the leaves black, resulting in a loss of antioxidant capacity that causes

green tea to have higher antioxidant properties than black tea (Pellegrini *et al*., 2003). This loss in antioxidant capacity was however not observed with the green and black tea in this study, where black tea had a higher TFC than green tea, and there was no significant difference regarding TPC of black and green tea. As only one source of green tea was analysed, this data is not representative of all green teas.

Table 3.2: Antioxidant content of fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea

Sample	Total Polyphenolic Content (mg GAE/g)	Total Flavonoid Content (mg CE/g)
Fermented		
Black Tea		
W	$24.56^{d} \pm 2.09$	$75.05^{b} \pm 2.61$
G	$26.85^{b} \pm 1.40$	$81.67^{e} \pm 3.00$
F	$28.50^{b} \pm 1.90$	$99.09^{f} \pm 1.21$
P	$33.00^{e} \pm 1.07$	$103.66^{g} \pm 1.50$
Average	**28.23 ± 3.57**	**89.87 ± 13.69**
Rooibos Tea		
F	$7.62^{a} \pm 0.30$	$49.64^{c} \pm 2.17$
L	$9.25^{a} \pm 0.34$	$69.57^{a} \pm 2.71$
P	$9.55^{a} \pm 0.37$	$58.70^{d} \pm 4.53$
Average	**8.81 ± 1.04**	**59.30 ± 9.98**
Unfermented-Controls		
Green Tea W	**$27.86^{b} \pm 1.32$**	**$71.67^{ab} \pm 3.24$**
Rooibos Tea L	**$12.09^{c} \pm 0.50$**	**$68.56^{a} \pm 0.39$**

Data is an average of 3 experiments ± SD. Mean values in each column with different letters are significantly different ($p \leq 0.05$).

Lower amounts of flavonoids, particularly aspalathin, are found in fermented Rooibos tea as a result of the oxidation processes of fermentation, resulting in a lower antioxidant capacity of fermented Rooibos tea than that of unfermented Rooibos tea (Standley *et al*., 2001). This trend was observed in this study, with unfermented Rooibos tea having a higher TPC and TFC than most fermented Rooibos teas. The results of previous studies (Record & Lane, 2001; Joubert *et al*., 2004; Joubert *et al*., 2005; Alarcón *et al*., 2008; Awoniyi *et al*., 2012; Bramati *et al*., 2003) followed the trend of high TPC for black and green tea and lower TPC for unfermented and fermented Rooibos tea. Henning *et al*. (2003) and von Gadow *et al*. (1997) had similar values for TPC for black tea (21.2-68.3 and 33.9mg/g GAE, respectively) and green tea (34.9 mg/g GAE,

von Gadow *et al*., 1997) compared to the present study which had an average TPC of 28.23 mg/g GAE for black tea and 27.86 mg/g GAE for green tea. A similar trend for the TFC was found in other studies on tea (von Gadow *et al*., 1997; Joubert *et al*., 2005; Bramati *et al*., 2003).

3.4.2 Antioxidant activity: DPPH, TEAC and ORAC – Camellia sinensis and Aspalathus linearis

Antioxidant activity can be measured using several different assays and these include ET and HAT based assays. In this study the ET assays DPPH and TEAC were used, and the HAT assay ORAC was used. ET assays measure the capacity of an antioxidant to reduce an oxidant, which results in a colour change when the oxidant is reduced, with the degree of change corresponding to the antioxidant's concentration (Zulueta *et al*., 2009). HAT based assays make use of competitive binding of antioxidants and substrates to thermally generated peroxyl radicals by means of decomposition of azo-compounds (Zulueta *et al*., 2009).

Green tea had the highest radical scavenging ability in the DPPH assay of all the teas (129.88 mg TE/g), followed by black tea (121.08-129.27 mg TE/g), unfermented Rooibos tea (64.30 mg TE/g), and lastly fermented Rooibos tea (39.86-52.82 mg TE/g) (Table 3.3).

Green tea and three of the black teas did not differ significantly in their radical scavenging ability, which is similar to a study done by Atoui *et al*. (2005). Von Gadow *et al*. (1997) also found in their study that green tea had a higher radical scavenging ability compared to black tea and Rooibos tea. Joubert *et al*. (2004) found that unfermented Rooibos tea had a higher radical scavenging ability than fermented Rooibos tea. Oxidation of the flavanols of green tea results in the lower radical scavenging ability of black tea (von Gadow *et al*., 1997). Polyphenol oxidases oxidize the powerful antioxidant gallocatechins, namely (+)-EGC and (+)-EGCG, due to their high oxidation potential and high concentration in green tea (von Gadow *et al*., 1997). The oxyproducts that are formed are thearubigens and theaflavins, which are the major phenolic compounds of black tea and are less potent antioxidants than the flavanols (von Gadow *et al*., 1997). Gallic acid is also present in black tea and is an effective hydrogen donor to the DPPH radical (von Gadow *et al*., 1997). The trend for TPC and TFC of the Rooibos teas correlate well with the radical scavenging abilities, indicating that higher antioxidant content of unfermented Rooibos tea results in its higher antioxidant activity compared to fermented Rooibos tea. The

major flavonoid in unfermented Rooibos tea is aspalathin, which is an active scavenger of DPPH. As a result of fermentation process, less than 7% of the aspalathin originally present is left in fermented Rooibos (von Gadow *et al*., 1997). Therefore fermentation decreases the radical scavenging ability of the aqueous extract and results in qualitative changes in phenolic composition and with oxidation a reduction in soluble total polyphenols (Joubert *et al*., 2004).

Du Toit, Volsteedt and Apostolides (2001) used DPPH generated antioxidant data to calculate the total radical scavenging capacity (RSC) of teas, which were then presented as vitamin C equivalents. Du Toit *et al*. (2001) showed that one or two cups of tea per day supply the equivalent of five portions of fruits and vegetables or 400 mg vitamin C equivalents in terms of RSC. Their results made use of an RSC equivalent to 200 mg of vitamin C as the daily intake. In this study, using averages for DPPH data (Table 3.4) it was calculated for black tea a RSC of 221.91 mg per cup and for Rooibos tea 81.02 mg per cup (Figure 3.2), which correlates well with the findings of du Toit *et al*. (2001). Roughly one cup of black tea and two cups of Rooibos tea would be sufficient to meet 200 mg vitamin C equivalents, antioxidant intake.

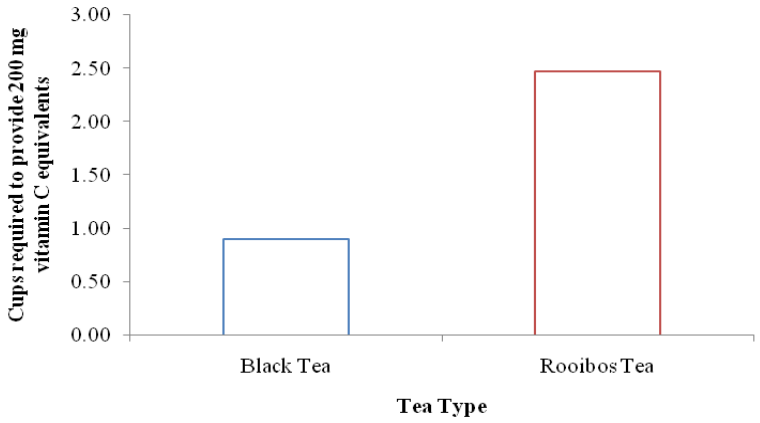

Figure 3.2: Number of cups (250 mL) of fermented *Camellia sinensis* and *Aspalathus linearis* tea required to provide an equivalent RSC of 200 mg vitamin C.

In the TEAC assay, black tea had the highest antioxidant activity (47.18-67.19 mg TE/g, 1887.20-2687.60 µmol/L), followed by green tea (51.25 mg TE/g, 2050.00 µmol/L), then unfermented Rooibos tea (29.87 mg TE/g, 1194.80 µmol/L). Fermented Rooibos tea had the lowest antioxidant activity (16.63-20.88 mg TE/g, 665.20-835.20 µmol/L).

As a result of the oxidation processes during fermentation of green tea, the catechin content is converted to oxyproducts such as thearubigins and theaflavins, and consequently there is a loss of antioxidant capacity (Pellegrini *et al*., 2003). EGCG is the predominant catechin in green tea (Ferruzzi, 2010), and one study found that when brewed for 4 minutes, green tea contained 1560 µmol/l compared to black tea which only contained 493 µmol/L (Record & Lane, 2001). This was however not the case in this study, in which black tea had a higher antioxidant activity compared to green tea, with a brewing time of 3 minutes in 200 ml distilled water for green and black tea. The total antioxidant activity has a good correlation with the TPC of the different teas. A similar trend for the higher total antioxidant activity of black compared to green tea was found in a study done by Gramza *et al*. (2005). Bramati *et al*. (2003) had a similar trend in their results for unfermented and fermented Rooibos tea as was found in this study, with unfermented Rooibos tea having a higher total antioxidant activity compared to fermented Rooibos tea. Green tea contains a high amount of catechins, whereas black tea contains lower amounts of catechins (Gramza *et al*., 2005). The higher antioxidant activity of black tea in the TEAC assay might be as a result of tannins, which constitutes 91.5% of the extract and has proven very strong antiradical properties (Gramza *et al*., 2005). An increase in the degree of esterification with gallates causes an increased scavenging activity of the radical cation $ABTS^{\bullet+}$ (Gramza *et al*., 2005).

In the ORAC assay, black tea had the highest antioxidant activity of all the teas (1162.10-1208.33 mg TE/g), followed by green tea (1123.11 mg TE/g), unfermented Rooibos tea (600.98 mg TE/g) and fermented Rooibos tea had the lowest antioxidant activity (555.37-608.00 mg TE/g).

This finding of high ORAC activity in black and green tea and lower activity in the fermented and unfermented Rooibos teas agrees with the studies done by Alarcón *et al*. (2008) and Awoniyi *et al*. (2012). These authors also found a good correlation between the high TPC in the green and black teas with high ORAC activity, and a lower TPC for Rooibos tea with lower ORAC activity. The fold differences between black and Rooibos tea related to antioxidant activity is 2.74, 2.95 and 2.06 for the DPPH, TEAC and ORAC assays respectively (Table 3.4) and reflects the calculated RSC.

Table 3.3: Antioxidant activity of fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea

Sample	DPPH (mg TE/g)	TEAC (mg TE/g)	ORAC (mg TE/g)
Fermented			
Black Tea			
W	121.08^{d} ± 6.76	47.18^{c} ± 1.95	1162.10^{bc} ± 20.61
G	123.16^{a} ± 5.99	50.03^{cd} ± 0.38	1208.33^{b} ± 33.85
F	129.27^{a} ± 5.34	61.48^{f} ± 2.42	1196.71^{b} ± 8.40
P	129.21^{a} ± 5.50	67.19^{g} ± 1.83	1204.21^{b} ± 2.58
Average	**125.68 ± 4.20**	**56.47 ± 9.45**	**1192.84 ± 21.05**
Rooibos Tea			
F	39.86^{b} ± 1.08	16.63^{a} ± 1.09	555.37^{a} ± 24.95
L	52.82^{c} ± 2.43	20.88^{b} ± 1.49	608.00^{a} ± 34.12
P	45.92^{bc} ± 1.39	19.63^{ab} ± 1.19	573.70^{a} ± 21.46
Average	**46.20 ± 6.48**	**19.05 ± 2.18**	**579.02 ± 26.72**
Unfermented-Controls			
Green Tea W	**129.88^{a} ± 4.27**	**51.25^{d} ± 3.74**	**1123.11^{c} ± 74.63**
Rooibos Tea L	**64.30^{e} ± 1.92**	**29.87^{e} ± 1.27**	**600.98^{a} ± 35.46**

Data is an average of 3 experiments ± SD. Mean values in each column with different letters are significantly different ($p \leq 0.05$). Antioxidant activity multiply by 4 to convert to mmol/L

Table 3.4: Summary of antioxidant content and activity of fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea expressed as per ml brewed tea

Sample	TPC (mg GAE/ml)	TFC (mg CE/ml)	DPPH (mg TE/ml)	TEAC (mg TE/ml)	ORAC (mg TE/ml)
Fermented					
Black Tea	$0.28^{a} \pm 0.02$	$0.90^{c} \pm 0.02$	$1.26^{a} \pm 0.06$	$0.56^{d} \pm 0.01$	$11.93^{b} \pm 0.08$
Rooibos Tea	$0.09^{b} \pm 0.00$	$0.59^{a} \pm 0.10$	$0.46^{b} \pm 0.06$	$0.19^{a} \pm 0.02$	$5.79^{a} \pm 0.27$
BT vs. RT (Fold difference)	3.11	1.53	2.74	2.95	2.06
Unfermented-Controls					
Green Tea W	$0.28^{a} \pm 0.01$	$0.72^{b} \pm 0.03$	$1.30^{a} \pm 0.04$	$0.51^{c} \pm 0.04$	$11.23^{b} \pm 0.75$
Rooibos Tea L	$0.12^{c} \pm 0.01$	$0.69^{ab} \pm 0.00$	$0.64^{b} \pm 0.02$	$0.30^{b} \pm 0.01$	$6.01^{a} \pm 0.35$

Data is an average of each tea type ± SD. Mean values in each column with different letters are significantly different ($p \leq 0.05$).
Antioxidant activity multiply by 4 to convert to mmol/L

3.4.3 Cellular and biological assays

3.4.3.1 NO scavenging ability

RNS are products formed when NO reacts with ROS, have been found to contribute to oxidative tissue/cellular damage (Tsai *et al*., 2007). By reducing NO through scavenging the rate at which RNS forms is reduced and consequently the degree of RNS induced cellular damage.

Black tea was the most effective at scavenging NO (70.38-73.62%), followed by green tea (66.39%), and unfermented Rooibos tea (44.58%) (Table 3.5). Fermented Rooibos tea was the least effective at scavenging NO (34.46-37.85%). Paquay *et al*. (2000) found that green tea had a much higher NO scavenging ability than black tea, which is the opposite of what was observed in this study.

Table 3.5: NO scavenging ability of fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea

Sample	NO Scavenged	
	(%)	**TE (µmol/L)**
Fermented		
Black Tea		
W	$70.38^{ac} \pm 2.53$	773.86
G	$71.19^{ac} \pm 1.30$	782.87
F	$73.12^{a} \pm 2.28$	804.03
P	$73.62^{a} \pm 1.36$	809.51
Average	**72.08 ± 1.54**	**792.57 ± 16.96**
Rooibos Tea		
F	$34.46^{b} \pm 3.91$	378.92
L	$37.80^{b} \pm 4.18$	415.67
P	$37.85^{b} \pm 4.18$	416.21
Average	**36.70 ± 1.94**	**403.60 ± 21.37**
BT vs. RT (Fold difference)	**1.96**	**1.96**
Unfermented-Controls		
Green Tea W	**66.39^{c} ± 4.43**	**730.03**
Rooibos Tea L	**44.58^{d} ± 5.21**	**490.18**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$).

Flavonoids act as antioxidants, and scavenge RNS and in doing so, may protect cells and plasma constituents against oxidative damage (Nijveldt *et al*., 2001; Coimbra *et al*., 2006). The trend observed for TFC in this study was similar to that of NO scavenging ability. It can

therefore be assumed that flavonoids are the major components responsible for scavenging NO, and as black tea and green tea are higher in flavonoid content than fermented and unfermented Rooibos tea, NO scavenging ability for black and green tea was thus also higher than that for fermented and unfermented Rooibos tea.

3.4.3.2 DCFH-DA

Cell-free, chemical assays such as ORAC/TEAC/DPPH measure potential antioxidant activity but do not take into account cellular absorption, distribution, metabolism and excretion. Therefore, the testing within an *in vitro* environment (in this study the Caco-2 cell line) gives a more realistic account of the cellular antioxidant activity, as the antioxidant components within a cellular environment either have a good antioxidant status or these molecules have a direct cellular effect.

At the dilutions used in this study, all tea samples were shown not to cause oxidative damage in the Caco-2 cell line (data not shown). The ability to quench radicals and thus reduce or prevent oxidative damage was investigated using the DCFH-DA assay. Green tea had the highest protection against oxidative damage (105.57%), followed by black tea (average of 96.72%), unfermented Rooibos (86.84%) and fermented Rooibos had the lowest protection with an average of 84.65% (Table 3.6). The reason why green tea had a value greater than 100% in protection is unknown. It could possibly be due to a pro-oxidative effect of green tea (Tao, Park & Lambert, 2014). A study done by Yoo *et al*. (2008) found that ROS generation was the lowest for green tea, followed by black tea and then Rooibos tea, meaning that green tea had the highest protection, followed by black tea and then Rooibos tea, which follows the trend found in this study. According to Yoo *et al*. (2008), flavanols have 5 to 8 OH groups which includes the *ortho*-(3′,4′) dihydroxy groups in the B-ring and 3-hydroxyl group and/or 3-galloyl group in the C-ring. The most of these is EGCG which has 8 OH groups and *ortho*-(3′,4′)-dihydroxy groups in the B-ring as well as a 3-galloyl group in the C-ring. Of the flavan-3-ols, predominantly catechin derivatives possess potent antioxidant activity. In the study done by Yoo *et al*. (2008), green tea contained the most EGCG among 17 herb varieties and it contained almost twice as much total catechin as black tea. Catechin had a lower radical scavenging activity than other catechin derivatives and therefore the authors concluded it is possible that EGCG is the principal compound that contributes to the high antioxidant activity of green tea (Yoo *et al*., 2008). Also present in black tea are the theaflavins and thearubigens that have also been shown to have antioxidant activity (Yang *et al*., 1998).

Table 3.6: Ability of fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea to protect Caco-2 cells from AAPH-induced oxidative damage

Sample	DCFH-DA (% Damage)
Fermented	
Black Tea	
W	$3.55^{a} \pm 0.14$
G	$3.33^{a} \pm 0.30$
F	$2.47^{a} \pm 0.22$
P	$3.75^{a} \pm 0.18$
Average	**3.28 ± 0.57**
Rooibos Tea	
F	$16.64^{e} \pm 1.54$
L	$14.74^{b} \pm 1.12$
P	$14.67^{b} \pm 1.07$
Average	**15.35 ± 1.11**
BT vs. RT (Fold difference)	**1.14**
Unfermented-Controls	
Green Tea W	**$-5.57^{c} \pm 0.62$**
Rooibos Tea L	**$13.16^{d} \pm 0.86$**
AAPH	**100**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$).

The antioxidant effects of tea on cell viability could be due to two mechanisms: direct antioxidant action by radical scavenging effect, and indirect antioxidant action by preventing intercellular ROS generation (Yoo *et al*., 2008). Tea polyphenols are potent scavengers of superoxide anions and hydroxy radicals (Roy *et al*., 2001) and activity of numerous enzymes is altered. Enzyme activity can either be inhibited by tea and tea polyphenols (Yang *et al*., 1998) or antioxidant enzymes such as glutathione peroxidase, glutathione reductase, catalase, quinone reductase, catalase and superoxide dismutase can be induced which effectively reduces the oxidative load and thereby minimising cellular damage (Roy *et al*., 2001).

Green tea has been found to have more EGCG, ECG, EGC, EC and C than black tea (Record & Lane, 2001; Yoo *et al*., 2008) which results in the higher antioxidant activity of green tea, and this can be seen in Table 3.6 where green tea had a greater protective effect against oxidative damage than black tea. Rooibos tea is significantly lower in EGCG, EGC, EC and C than green and black tea (Yoo *et al*., 2008). Aspalathin is the major flavonoid of unfermented Rooibos tea, 93% of which is converted to other active compounds during the fermentation process (von Gadow *et al*., 1997). These active compounds include dihydro-

orientin, dihydro-iso-orientin, orientin, iso-orientin and polymeric products (von Gadow *et al*., 1997).

The fold difference between NO scavenging activity and the cellular protective effects of black tea was 1.96 and 1.14 higher than for Rooibos tea, respectively.

3.5 Conclusion

The antioxidant content namely the TPC and TFC of fermented *Camellia sinensis* was 3.11 and 1.53 times greater than fermented *Aspalathus linearis* tea. The measured antioxidant activity was also 2.74, 2.95 and 2.06 times greater for fermented *Camellia sinensis* compared to fermented *Aspalathus linearis* tea when determined using the DPPH, TEAC, and ORAC assays. Both types of fermented teas scavenged NO and this effect was 1.96 times better for fermented *Camellia sinensis*. Both tea types effectively protected Caco-2 cells against AAPH-induced oxidative damage, again the effect of fermented *Camellia sinensis* was 1.14 fold greater than fermented *Aspalathus linearis* tea.

Chapter 4: Effect of simulated adult GIT digestion on the antioxidant properties of *Camellia sinensis* and *Aspalathus linearis* teas

4.1 Abstract

The antioxidant potential of radical scavengers in tea is dependent on digestion conditions that results in biotransformation of flavanols and flavonoids in the stomach, small intestine and colon of the GIT. Antioxidant content and activity of *Camellia sinensis* and *Aspalathus linearis* teas and their *in vitro* adult digests (Stomach digest (SD) and SD followed by duodenal digestion (SDD)) were determined. The ability to scavenge NO, and cellular antioxidant activity in the Caco-2 cell line was determined. TFC of *Camellia sinensis* teas remained relatively stable throughout digestion. TPC, DPPH and ORAC values for *Camellia sinensis* teas decreased significantly following duodenal digestion (SDD). Stomach digestion (SD) decreased DPPH, TEAC, ORAC values of *Camellia sinensis* teas. TPC of *Camellia sinensis* teas increased with SD. *Aspalathus linearis* teas decreased significantly following SDD in terms of DPPH, TEAC, and ORAC values. Fermented Rooibos tea increased in TPC following SD. Alkaline pH and SDD decreased NO scavenging ability and protection in the DCFH-DA assay of *Camellia sinensis* and *Aspalathus linearis* teas, whereas acidic pH and SD had a less significant effect. After digestion *Camellia sinensis* teas provided more than 83% protection, while *Aspalathus linearis* teas provided 71% and higher protection. *Camellia sinensis* and *Aspalathus linearis* teas provided significant protection during and after digestion, despite losses in antioxidant content and activity, showing that these teas are vital and functional sources of antioxidants that could be incorporated in a healthy, balanced diet.

4.2 Introduction

Tea is widely consumed by all South African communities (Labadarios *et al*., 2005) and is an inexpensive commodity that is shelf-stable and readily available throughout South Africa. Water is boiled in the preparation of tea, thereby improving the quality of water and making it safer for consumption (Rufener *et al*., 2010).

Low antioxidant status of the South African population is currently a major concern, and is believed to be as a result of inadequate consumption of fruits and vegetables (Smuts *et al*., 2005; Vorster *et al*., 1997). Tea is a major source of antioxidants (Chun *et al*., 2007; von Gadow *et al*., 1997) which can used to help address poor antioxidant status, but cannot replace vegetables and fruit as a source of nutrients such as vitamins.

Although a product such as tea has a high antioxidant content and activity, it is essential to determine if this activity is retained following GIT transit. Antioxidant potential is dependent on digestion conditions that can cause the biotransformation of flavanols and other flavonoids in the stomach, small intestine and colon of the GIT (Spencer, 2003). Changes in activity can be the result of pH changes and/or the effect of digestive enzymes. Contradictory findings have been reported related to the effect of pH. Studies have found that acidic pH conditions can either decrease antioxidant activity of tea (Record & Lane, 2001), or have no significant effect (Viljoen, 2008; Neilson *et al*., 2007). Numerous studies have observed that tea antioxidants are particularly affected by near neutral conditions (Ferruzzi, 2010; Spencer, 2003; Green *et al*., 2007; Viljoen, 2008). These studies propose that polyphenolic compounds are relatively stable under acidic conditions, however under neutral and alkaline conditions they degrade in pH driven auto-oxidation type reactions. Oxidised products sometimes possess greater radical scavenging activity than their original flavonoids (Spencer, 2003).

Little is known about how digestion in each compartment i.e. the stomach and duodenum, of the digestion process affects antioxidant content and activity of both *Camellia sinensis* and *Aspalathus linearis* fermented tea. Whether this change in activity is solely pH dependent or does the presence of the digestive enzymes make a significant contribution to measured activity is unknown. Although, Breiter *et al*. (2011) have reported that only 0.26% of the total amount of ingested Rooibos flavonoids were present in the plasma following ingestion, however polyphenolics play an important role in the health of the GIT (Gee & Johnson, 2001). Therefore it is important to determine the effect of GIT digestion on the antioxidant content and activity and cellular protection of *Aspalathus linearis* against oxidative damage.

The purpose of this study was to determine, using a simulated *in vitro* digestion model, the effect of GIT digestion on the antioxidant content and activity of *Camellia sinensis* and *Aspalathus linearis* teas. Furthermore, the effect of digestion on the ability of tea to scavenge NO thereby preventing the formation of RNS was also evaluated. Lastly, the cellular antioxidant activity of *Camellia sinensis* and *Aspalathus linearis* tea digests in a physiologically relevant cell culture model was also determined.

4.3 Materials and Methods

4.3.1 In vitro simulated adult gastrointestinal digestion

All tea samples were the same as used in Chapter 3. Tea extracts were prepared as described in Chapter 3, section 3.3. Samples were then digested using an *in vitro* digestion model that simulates adult digestion (Figure 4.1). To simulate gastric digestion in the stomach, the pH of the samples were adjusted to pH 2 with 1 M HCl before 5 µl pepsin (from porcine gastric mucosa, Sigma-Aldrich) (20 mg/ml) per ml of sample was added to each sample (except pH controls) and incubated for 30 min in 37°C water bath to simulate stomach digestion (SD). pH controls for stomach digestion (pH C-SD) were subjected to the same process with pepsin omitted. A SD and pH C-SD sample was collected. The pH of the remaining samples were adjusted to pH 7 with 1 M $NaHCO_3$, then 5 µl pancreatin (from porcine pancreas, Sigma-Aldrich) (4 mg/ml) per ml of sample was added (except pH control) and incubated for 60 min in 37°C water bath to simulate duodenal digestion. This was known as stomach and duodenal digests (SDD). pH controls for stomach digestion (pH C-SD) were subjected to the same process with pancreatin omitted (pH C-SDD). To terminate enzymatic activity all samples were boiled for 5 min at 95°C and stored at -20°C until needed. Dilutions used for antioxidant content and activity, NO and cellular protective methodologies are the same as described in Chapter 3, section 3.3. All stock solutions were stored at -20°C in the dark until further analysis.

Antioxidant content was determined using the Folin-Ciocalteu and aluminium chloride assays, as described in Sections 3.3.2 and 3.3.3. The antioxidant activity of each sample was determined using the DPPH, TEAC and ORAC assays as described in Sections 3.3.4-3.3.6. Likewise NO scavenging activity and cellular protective effects against AAPH-induced oxidative damage was determined as described in Sections 3.3.7-3.3.9.

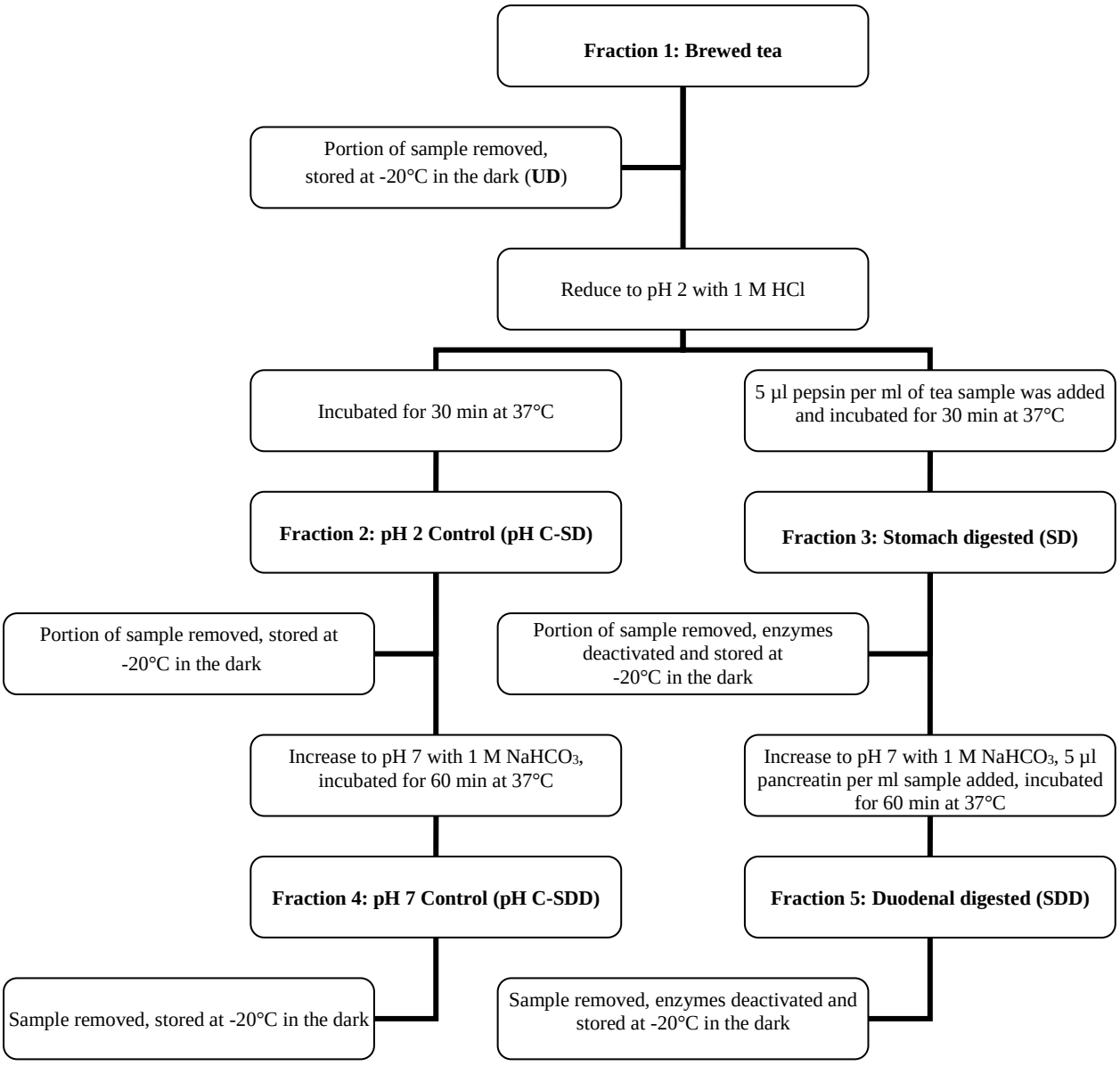

Figure 4.1: Methodology flow-chart of *in vitro* adult simulated gastrointestinal digestion

4.3.2 Statistical analysis

The means were subjected to one-way ANOVA, calculated at the 5% level using STATISTICA Version 8 to compare treatment means for total polyphenol and flavonoid content, DPPH radical scavenging method, and TEAC, ORAC, NO scavenging and DCFH-DA assays. One-way ANOVA was used when more than two sample means were compared.

% Change was calculated the following way:

$$\frac{\textbf{Undigested} - \textbf{Treatment (e.g. digest or control)}}{\textbf{((Undigested + Treatment)} \div \textbf{2)}} \times \textbf{100 = \% Change}$$

4.4 Results and Discussion

In the stomach, pepsin digests protein at pH 2. After a digestion period of about 30 minutes, food moves from the stomach to the duodenum where the pancreas releases proteases, lipase and carbohydrase at pH 7 (Whitney & Rolfes, 2008, p. 77). There are several model systems to evaluate the effect of gastric and small intestinal digestion, such as static monocompartmental models, dynamic monocompartmental models, and dynamic bi- and multicompartmental models (Guerra *et al*., 2012). The first level of testing is using a simplistic laboratory model where each component contributing to digestion is controlled. Using a laboratory based model the effect of adult digestion on the TPC and TFC content of tea was determined. Likewise, the effect of digestion on antioxidant activity was determined using the DPPH, TEAC and ORAC assays. In addition the effect of digestion on the NO scavenging and cellular protective effects of the tea digests was determined.

4.4.1 Antioxidant content

4.4.1.1 TPC and TFC – Camellia sinensis

TPC of undigested black tea used in this study varied from 24.56-33.00 mg GAE/g. For pH C-SD the TPC was 27.09-41.99 mg GAE/g with a 9.80-23.98% increase extraction of polyphenolics at pH 2. This effect was statically significant for all black tea samples and was only biologically relevant for G, F and P (18.69-23.98%). No difference in the TPC was found between UD and pH C-SD for green tea. With the addition of digestive enzymes, TPC was 28.01-38.98 mg GAE/g for SD (Table 4.1). The differences in TPC for pH C-SD compared to SD was not significant for W and G but was significant for F and P with a decrease in TPC from 23.12% compared to 15.86% and 23.98 compared to 16.62% respectively. In general with SD, TPC levels increased significantly by 10.92-16.62% while for green tea SD had no effect on TPC.

For black tea, TFC remained relatively constant with 75.05-103.66 mg CE/g, 73.66-106.82 mg CE/g and 73.92-108.79 mg CE/g for undigested, SD and SDD respectively. TFC of pH C-SD and SD were not significantly different for W and G but were for F and P. For the latter two samples the effect was less than 10%. For pH C-SDD, TFC levels were significantly less than pH C-SD with a decrease compared to UD of 7.03-19.31%. With the inclusion of pancreatin enzymes the loss of activity was significantly less than pH C-SDD and compared to UD was not significant.

Table 4.1: Antioxidant content of undigested and adult digested fermented and unfermented *Camellia sinensis* tea

Sample		Total Polyphenolic Content		Total Flavonoid Content	
		mg GAE/g	% Change	mg CE/g	% Change
Fermented					
W	UD	$24.56^{def} \pm 2.09$	–	$75.05^{fg} \pm 2.61$	–
	SD	$28.01^{hi} \pm 1.44$	↑ **15.22**	$73.66^{ef} \pm 2.03$	↓ 1.87
	pH C-SD	$27.09^{gh} \pm 2.75$	↑ 9.80	$74.81^{efg} \pm 1.42$	↓ 0.32
	SDD	18.29ab ± 0.18	↓ 29.26s	73.92ef ± 0.83	↓ 1.52ns
	pH C-SDD	$18.59^{ab} \pm 0.73$	↓ **27.67**	$69.95^{cd} \pm 2.46$	↓ 7.03
G	UD	$26.85^{fgh} \pm 1.40$	–	$81.76^{i} \pm 3.00$	–
	SD	$29.95^{ij} \pm 1.41$	↑ **10.92**	$80.27^{hi} \pm 1.40$	↓ 1.84
	pH C-SD	$32.38^{jk} \pm 2.23$	↑ **18.67**	$80.49^{hi} \pm 1.43$	↓ 1.57
	SDD	$17.75^{a} \pm 0.27$	↓ **40.81**^{s}	$77.84^{gh} \pm 0.58$	↓ 4.91^{ns}
	pH C-SDD	$17.49^{a} \pm 0.40$	↓ **42.22**	$69.39^{cd} \pm 2.14$	↓ **16.38**
F	UD	$28.50^{hi} \pm 1.90$	–	$99.09^{k} \pm 1.21$	–
	SD	$33.41^{k} \pm 0.64$	↑ **15.86**	$90.30^{j} \pm 1.89$	↓ 9.28
	pH C-SD	$35.95^{l} \pm 1.48$	↑ **23.12**	$91.17^{j} \pm 2.02$	↓ 8.33
	SDD	$22.12^{cd} \pm 0.84$	↓ **25.20**^{s}	$92.74^{j} \pm 0.92$	↓ 6.62^{ns}
	pH C-SDD	$20.68^{bc} \pm 0.57$	↓ **31.80**	$81.23^{i} \pm 2.51$	↓ **19.81**
P	UD	$33.00^{k} \pm 1.07$	–	$103.66^{l} \pm 1.50$	–
	SD	$38.98^{m} \pm 0.75$	↑ **16.62**	$106.82^{lm} \pm 3.37$	↑ 3.00
	pH C-SD	$41.99^{n} \pm 4.16$	↑ **23.98**	$107.12^{m} \pm 2.22$	↑ 3.28
	SDD	$25.21^{efg} \pm 0.68$	↓ **26.77**^{s}	$108.79^{m} \pm 1.73$	↑ 4.83^{ns}
	pH C-SDD	$23.76^{de} \pm 0.84$	↓ **32.56**	$90.13^{j} \pm 2.68$	↓ **19.96**
Unfermented-Control					
W	UD	$27.86^{hi} \pm 1.32$	–	$71.67^{de} \pm 3.24$	–
	SD	$26.02^{efgh} \pm 0.84$	↓ 6.83	$65.40^{b} \pm 1.53$	↓ 9.15
	pH C-SD	$27.45^{gh} \pm 1.96$	↓ 1.48	$68.10^{bc} \pm 0.74$	↓ 5.11
	SDD	$16.19^{a} \pm 0.39$	↓ **52.99**^{s}	$70.05^{cd} \pm 0.91$	↓ 2.29^{s}
	pH C-SDD	16.43a ± 0.38	↓ **51.61**	61.61a ± 1.31	↓ 15.10

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

For green tea, the TFC of pH C-SD and SD compared to UD significantly reduced by 5.11% and 9.15% respectively. As for black tea, green tea pH C-SDD reduced TFC by 15.10% while for SDD this decrease was only 2.29%.

Phenolic and flavonoid content was not drastically affected by low pH and stomach digestion of tea in this study, similar to what other studies have shown (Cilliers & Singleton, 1989; Record & Lane, 2001; Green *et* al., 2007; Neilson *et al*., 2007). Record and Lane (2001) found that catechins GA, EGC, EGCG, EC and ECG in green tea were slightly lowered when incubated at pH 2 for 1 hour, while GCG increased slightly, whereas in black tea, EGC, EGCG, GCG, and ECG were slightly lowered when incubated at pH 2 for 1 hour. In contrast, all green and black tea catechins were drastically reduced when incubated at pH 7 for 15 to 60 minutes. This showed that various catechins, especially EGCG and EGC, were unstable and degraded under neutral/alkaline conditions (Record & Lane, 2001). At elevated pH (6-7.5), catechins are sensitive and degrade in pH driven auto-oxidation type reactions (Ferruzzi, 2010). Yoshino *et al*. (1999) found that EGCG content decreased to 19.4% when exposed to intestinal juice for 5 minutes, due to the alkalinity of the substance. Neilson *et al*. (2007) observed that EGCG, EGC, and ECG degraded during *in vitro* intestinal digestion with 64.4-85.9, 66.8-88.4, and 54.0-55.1%, respectively, while EC and C had losses of 7.6-10.3 and 7.0-8.2%, respectively.

To summarise, with SD of black tea an increase in the extraction of polyphenolics was observed which with further digestion decreases by 28.57% compared to UD. No changes in TFC levels were found following digestion.

4.4.1.2 TPC and TFC – Aspalathus linearis

The effect of digestion on the TPC and TFC of fermented Rooibos tea was evaluated (Table 4.2). With SD TPC increased to 9.28-10.86 mg GAE/g and this increase was statistically significant for F and L. For Rooibos tea P, the TPC decreased, although not significant. For all Rooibos teas the reduction of pH to 2 caused a significant increase in TPC. Differences between SD and pH C-SD was not significant for teas F, but was for tea P and L. With further digestion the TPC decreased to 7.98-9.76 mg GAE/g for SDD, which was not significantly different from the UD. This change was due to the effect of pH and for all tea types the differences between SDD and pH C-SDD (7.78-9.32 mg GAE/g) was not significant. For unfermented Rooibos tea, TPC of SD and pH C-SD did not differ significantly from undigested tea, but with duodenal digestion a significant decrease in TPC due to pH was found for SDD and pH C-SDD. In summary (Table 4.5) the TPC of Rooibos tea increased by 10.53% for SD and then decreased after SDD, however the final value was not statistically different from the undigested value.

The TFC of the Rooibos tea digests were then evaluated. The TFC of UD fermented Rooibos tea was 49.64-69.57 mg CE/g. With SD a significant increase for teas F in TFC was found but TFC levels were significantly less for L and P. The range for TFC of SD was 45.65-63.48 mg CE/g for fermented Rooibos tea. Change in pH associated with pH C-SD resulted in a 10.29-24.93% decrease in TFC. For fermented Rooibos tea, differences between SD and pH C-SD was not significant, except for sample P, indicating the decrease in TFC was due to pH changes. For all samples, compared to UD, TFC levels for SDD (45.82-58.70 mg CE/g) were significantly less than UD. Differences between SDD and pH C-SDD were significant for all digests indicating that other factors besides pH alone is responsible for changes in TFC.

For unfermented Rooibos tea, TFC for SD and SDD was significantly less than UD, with a decrease of 20.24% and 13.82% respectively. This decrease in TFC was not due to pH as changes in pH caused a 44.12% and a 25.99% decrease in TFC for pH C-SD and pH C-SDD, respectively. Indications are that the presence of proteins such as pepsin and pancreatin protects against flavonoid degradation.

To summarise (Table 4.5) with simulated GIT digestion in the stomach, TFC decreased by 20.56% and following SDD, TFC decreased by 10.71% compared to UD. This implies that when SD is compared to SDD, levels of TFC is reduced in the stomach but then is increased in the duodenum and this effect was not due to pH changes.

Fermented Rooibos tea contains lower amounts of flavonoids, particularly aspalathin, due to the oxidation processes of fermentation. This results in a lower antioxidant capacity of fermented Rooibos tea than that of unfermented Rooibos tea (Standley *et al*., 2001). Although only one unfermented Rooibos tea sample was used in this study as a control, unfermented Rooibos tea had a higher TPC and TFC than fermented Rooibos tea in most treatments. At acidic pH of 3, 4 and 5, Viljoen (2008, pp.177-178) found that aspalathin, iso-orientin and orientin levels of fermented and unfermented Rooibos tea remained unchanged. In this study at pH 2 following 30 minutes exposure at 37°C a 10.29-25.01% and 44.12% decrease in TFC levels was measured for fermented and unfermented Rooibos tea respectively. Cilliers and Singleton (1989) did however state that the oxidation process is slow under acidic conditions, which suggests that even though the process might be slow, it does occur under acidic conditions.

Table 4.2: Antioxidant content of undigested and adult digested fermented and unfermented *Aspalathus linearis* tea

Sample		Total Polyphenolic Content		Total Flavonoid Content	
		mg GAE/g	% Change	mg CE/g	% Change
Fermented					
F	UD	$7.62^{a} \pm 0.30$	–	$49.64^{fg} \pm 2.17$	–
	SD	$9.28^{cde} \pm 0.32$	↑ **19.64**	$50.56^{bc} \pm 1.12$	↑ 1.84
	pH C-SD	$9.88^{e} \pm 1.18$	↑ **25.83**	$44.78^{abc} \pm 0.61$	↓ **10.29**
	SDD	$7.98^{ab} \pm 0.25$	↑ 4.62^{s}	$45.82^{cde} \pm 0.53$	↓ 8.00^{ns}
	pH C-SDD	$7.78^{ab} \pm 0.14$	↑ 2.08	$42.01^{a} \pm 0.95$	↓ **16.65**
L	UD	$9.25^{cde} \pm 0.34$	–	$69.57^{k} \pm 2.71$	–
	SD	$10.86^{f} \pm 0.46$	↑ **16.01**	$63.48^{i} \pm 1.14$	↓ 9.15
	pH C-SD	$12.43^{h} \pm 1.42$	↑ **29.34**	$54.15^{hi} \pm 1.42$	↓ **24.93**
	SDD	$9.76^{e} \pm 0.46$	↑ 5.37^{s}	$58.70^{j} \pm 1.37$	↓ **16.95**^{s}
	pH C-SDD	$9.32^{cde} \pm 0.04$	↑ 0.75	$51.38^{gh} \pm 1.04$	↓ **30.08**
P	UD	$9.55^{de} \pm 0.37$	–	$58.70^{j} \pm 4.53$	–
	SD	$9.38^{cde} \pm 0.49$	↓ 1.80	$45.65^{bcd} \pm 0.96$	↓ **25.01**
	pH C-SD	$11.35^{fg} \pm 1.06$	↑ **17.22**	$48.22^{ef} \pm 0.97$	↓ **19.60**
	SDD	$8.69^{bcd} \pm 0.31$	↓ 9.43^{ns}	$54.91^{i} \pm 0.06$	↓ 6.67^{s}
	pH C-SDD	$8.48^{abc} \pm 0.25$	↓ **11.87**	$47.60^{def} \pm 0.79$	↓ **20.88**
Unfermented-Control					
L	UD	$12.09^{gh} \pm 0.50$	–	$68.56^{k} \pm 0.39$	–
	SD	$11.75^{fgh} \pm 0.35$	↓ 2.85	$55.96^{ab} \pm 1.98$	↓ **20.24**
	pH C-SD	$12.29^{gh} \pm 0.68$	↑ 1.64	$43.78^{abc} \pm 0.50$	↓ **44.12**
	SDD	$8.21^{ab} \pm 0.17$	↓ **38.23**^{s}	$59.70^{j} \pm 1.11$	↓ **13.82**^{s}
	pH C-SDD	$8.73^{bcd} \pm 0.10$	↓ **32.28**	$52.79^{hi} \pm 1.14$	↓ **25.99**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)

It is possible that certain flavonoids are more sensitive to very low pH conditions, and therefore degrade under such conditions. Rodríguez-Roque *et al*. (2013) did an *in vitro* digestion of blended fruit juices, and noted that during gastric digestion, phenolic compounds show different stabilities. Overall the concentration of phenolic compounds increased, which was reported to be due to phenolic release following hydrolysis by the low pH and enzyme action. This could account for the 1.84% and 3.04% increase in TFC seen with teas F and P, and the 19.64% and 16.01% increase in TPC found with teas F and L, respectively. Gumienna, Lasik and Czarnecki (2011) observed that during stomach digestion of red grape

wine and chokeberry wine, TPC decreased slighly however significantly, while white grape wine remained stable.

In contrast, oxidation of phenolic compounds occurs quite rapidly at higher pH (Cilliers & Singleton, 1989) and this can lead to the formation of compounds with either reduced or increased antioxidant activity (Spencer, 2003). Viljoen (2008, pp.177-178) reported significant decreases in the aspalathin, iso-orientin and orientin content of fermented and unfermented Rooibos tea at pH 7 and temperatures between 30 and 40°C. This was similar to what was found in this study, where Rooibos tea was subjected pH 7 for 60 minutes at 37°C, which resulted in a decrease of 16.65-30.08% and 25.99% for TFC of fermented and unfermented Rooibos tea, respectively. Interestingly for both SD and SDD the presence of protein such as pepsin and pancreatin reduced the impact of pH effects. Rodríguez-Roque *et al*. (2013) observed in their study that flavonoid concentration was significantly decreased during small intestinal digestion (41%), while phenolic acids decreased by 14%. According to Rodríguez-Roque *et al*. (2013) this was as a result of oxidation and polimerization reactions that occur under alkaline pH conditions, causing phenolic derivatives to be formed, such as chalcones. Due to the high molecular weight of these compounds, they are unavailable for absorption. Gumienna *et al*. (2011) found that after stomach digestion, red grape wine increased in TPC with small intestinal digestion, whereas white grape and chokeberry wines decreased in TPC.

4.4.2 Antioxidant activity of Camellia sinensis: DPPH, TEAC and ORAC

The antioxidant activity of *Camellia sinensis* tea was determined using DPPH, TEAC and ORAC assays respectively. Antioxidant activity as well as the change in activity following stomach and duodenal digestion is presented in Table 4.3.

4.4.2.1 DPPH

In black tea the antioxidant scavenging activity of undigested tea measured with the DPPH assay was 121.08-129.27 mg TE/g, which with SD decreased significantly to 83.07-98.41 mg TE/g (Table 4.3). With SDD this decreased to 45.92-85.73 mg TE/g, with a percentage decrease of 31.19% and 62.50% for SD and SDD respectively (Table 4.5). Differences between undigested and pH C-SD was 52.66-60.99%, showing that a decrease in pH causes a significant decrease in antioxidant activity however in the presence of pepsin this effect was only 27.06-37.24%. Likewise, for SDD the change in pH caused a total loss of antioxidant activity of 89.00-128.36%. As for SDD in the presence of pancreatin, this effect is less

compared to UD and the decrease in activity was 40.46-92.37%. The same effect was observed for the unfermented green tea control. All changes were biologically relevant.

Nanjo *et al*. (1996) found that scavenging effects of tea catechins were ten times lower at pH 4 than at pH 7, particularly that of (+)-C and (-)-EC. Another study by Wootton-Beard *et al*. (2011) found that antioxidant capacity as measured by the DPPH assay of vegetable juices increased slightly during gastric digestion, and then decreased slightly during duodenal digestion. Chen *et al*. (2013) reported that antioxidant activity as measured by the DPPH assay of tea-containing juices increased significantly with gastric digestion, and then decreased significantly during duodenal digestion. According to Chen *et al*. (2013), this could be as a result of structural transformation in the polyphenols. The pH stability of catechins could explain the differences between SD and SDD. Zhu *et al*. (1997) reported that in neutral to alkaline solutions, catechins are extremely unstable and are degraded. In contrast in acidic conditions, catechins are fairly stable. In this study it was observed that even though a significant decrease in radical scavenging ability occurred during SD and acidic conditions, the most drastic decrease was seen during SDD and neutral pH conditions. Interestingly in the presence of digestive enzymes this decrease in antioxidant activity measured with the DPPH assay was less. It is also possible that polyphenolic compounds either bind to digestive enzymes, thereby inactivating them and lowering the antioxidant capacity of the tea solutions, and/or protects against pH induced degradation (Bandyopadhyay *et al*., 2012). Enzyme proteins may also have scavenged free radicals by mopping them up (Roche *et al*., 2008), which in this study resulted in the measurement of higher antioxidant activities for SD and SDD than for pH C-SD and pH C-SDD, respectively.

In summary, antioxidant activity of digested black tea measured with the DPPH assay decreased by 31.19% for SD and then a further 31.31% for SDD with 37.50% activity remaining following SDD.

4.4.2.2 TEAC

Antioxidant activity measured with the TEAC assay of UD black tea was 47.18-67.19 mg TE/g, which decreased with SD to 30.95-55.25 mg TE/g and with SDD to 40.70-68.42 mg TE/g. This is an average decrease of 24.00 and 5.50% for SD and SDD respectively. Differences between UD and SDD were not significant. For pH C-SD antioxidant activity was increased by 8.75-11.98%, which was significant for all tea types except G. The effect of SDD caused an increase in antioxidant activity following SD, the values of which were 1.81-

20.57% different from the values of the UD teas. For pH C-SDD, pH 7 alone caused a 7.11-34.00% decrease in activity. The antioxidant activity of UD green tea was 51.25 mg TE/g, and showed a similar trend to that of black tea. Antioxidant activity decreased from 51.25 mg TE/g to 38.97 mg TE/g for SD and a further loss in activity to 38.47 mg TE/g was found for SDD. Some of the changes in activity were biologically relevant, e.g. SD for W.

Table 4.3: Antioxidant activity of undigested and adult digested fermented and unfermented *Camellia sinensis* tea

Sample		DPPH		TEAC		ORAC	
		mg TE/g	% Change	mg TE/g	% Change	mg TE/g	% Change
Fermented							
W	UD	121.08^{n} ± 6.76	–	47.18^{fg} ± 1.95	–	1162.10^{fgh} ± 20.61	–
	SD	83.07^{ijk} ± 1.22	↓ **37.24**	30.95^{a} ± 2.94	↓ **41.55**	1005.37^{cd} ± 79.27	↓ **14.46**
	pH C-SD	64.49^{f} ± 5.91	↓ **60.99**	53.19^{hij} ± 4.75	↑ **11.98**	1211.68^{h} ± 8.91	↑ 4.18
	SDD	55.78^{e} ± 5.06	↓ **73.84**^{s}	44.40^{ef} ± 2.66	↓ 6.07^{s}	841.60^{b} ± 90.25	↓ **31.99**^{s}
	pH C-SDD	42.46^{bc} ± 2.12	↓ **96.15**	43.94^{def} ± 4.77	↓ 7.11	1186.55^{gh} ± 14.58	↑ 2.08
G	UD	123.16^{no} ± 5.99	–	50.03^{ghi} ± 0.38	–	1208.33^{h} ± 33.85	–
	SD	89.30^{k} ± 3.50	↓ **31.87**	40.90^{cde} ± 3.67	↓ **20.08**	1065.28^{def} ± 94.08	↓ **12.58**
	pH C-SD	69.69^{fg} ± 5.62	↓ **55.45**	54.61^{ij} ± 3.66	↑ 8.75	1218.18^{h} ± 1.51	↑ 0.81
	SDD	45.92^{cd} ± 5.79	↓ **91.37**^{s}	40.70^{bcde} ± 3.34	↓ **20.57**^{ns}	550.41^{a} ± 53.33	↓ **74.82**^{s}
	pH C-SDD	26.87^{a} ± 1.36	↓ **128.36**	35.49^{ab} ± 2.59	↓ **34.00**	1187.31^{gh} ± 12.65	↓ 1.75
F	UD	129.27^{o} ± 5.34	–	61.48^{k} ± 2.42	–	1196.71^{h} ± 8.40	–
	SD	98.23^{l} ± 3.08	↓ **27.29**	48.18^{fgh} ± 1.90	↓ **24.26**	1011.30^{cd} ± 90.25	↓ **16.79**
	pH C-SD	72.40^{gh} ± 5.84	↓ **56.40**	67.11^{l} ± 5.14	↑ 8.76	1215.14^{h} ± 6.48	↑ 1.53
	SDD	77.05^{ghi} ± 2.47	↓ **50.62**^{s}	57.38^{jk} ± 1.50	↓ 6.90^{s}	913.70^{bc} ± 117.10	↓ **26.82**^{ns}
	pH C-SDD	37.45^{b} ± 0.50	↓ **110.15**	44.22^{ef} ± 2.83	↓ **32.66**	1202.84^{h} ± 4.13	↑ 0.51
P	UD	129.21^{o} ± 5.50	–	67.19^{l} ± 1.83	–	1204.21^{h} ± 2.58	–
	SD	98.41^{l} ± 2.77	↓ **27.06**	55.25^{ij} ± 1.57	↓ **19.50**	1093.69^{defg} ± 85.28	↓ 9.62
	pH C-SD	75.35^{gh} ± 5.82	↓ **52.66**	74.18^{m} ± 3.53	↑ 9.89	1219.62^{h} ± 0.94	↑ 1.27
	SDD	85.73^{jk} ± 4.62	↓ **40.46**^{s}	68.42^{l} ± 2.18	↑ 1.81^{s}	948.40^{c} ± 108.06	↓ **23.77**^{s}
	pH C-SDD	49.63^{cde} ± 1.38	↓ **89.00**	52.96^{hij} ± 3.56	↓ **23.69**	1207.89^{h} ± 4.18	↑ 0.31
Unfermented-Control							
W	UD	129.88^{o} ± 4.28	–	51.25^{ghi} ± 3.74	–	1123.11^{efgh} ± 74.63	–
	SD	106.16^{m} ± 5.93	↓ **20.10**	38.97^{bcd} ± 3.32	↓ **27.22**	1054.85^{de} ± 107.36	↓ 6.27
	pH C-SD	79.65^{hij} ± 6.83	↓ **47.95**	48.15^{fgh} ± 4.95	↓ 6.24	1205.74^{h} ± 5.19	↑ 7.10
	SDD	51.46^{de} ± 4.02	↓ **86.49**^{s}	38.47^{bc} ± 2.89	↓ **28.49**^{ns}	631.96^{a} ± 71.91	↓ **55.97**^{s}
	pH C-SDD	42.67^{bc} ± 1.33	↓ **101.08**	30.81^{a} ± 2.44	↓ **49.82**	1203.44^{h} ± 18.15	↑ 6.91

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)
Antioxidant activity multiply by 4 to convert to mmol/L

In summary for black tea, digestion caused a 24.00% decrease for SD in antioxidant activity, which increased by 18.50% following SDD (Table 4.5).

The TEAC assay is usually the most commonly used assay for the determination of antioxidant activity of food products as this assay is stable to pH and can be used to study pH effect on activity (Zulueta *et al*., 2009). Chen *et al*. (2013) reported that the antioxidant activity of tea-containing juices as measured by the TEAC assay decreased following gastric and duodenal phases of digestion. This implies that pH, regardless of being acidic or neutral, affects the antioxidant activity of the polyphenolic compounds present in tea due to pH dependent structural transformation which causes loss of activity. The only exception to this was the stomach control pH 2 treatment of black tea, which increased in antioxidant activity. Wootton-Beard *et al*. (2011) observed that following *in vitro* gastric digestion of fruit juice that the inhibitory effects increased significantly. These authors suggest that pH may cause racemisation of polyphenolics resulting in antioxidants that are more reactive early in the digestion process, especially at acidic pH in the gastric phase and less reactive in the duodenal phase. Gumienna *et al*. (2011) observed in their study that with stomach digestion there was an increase in antioxidant activity of white and red grape wine with the TEAC assay, while chokeberry wine decreased during stomach digestion. Following small intestinal digestion, all three wines decreased in antioxidant activity.

4.4.2.3 ORAC

The antioxidant activity measured with the ORAC assay of UD black tea was 1162.10-1208.33 mg TE/g which decreased to 1005.37-1093.69 mg TE/g for SD and 550.41-948.40 mg TE/g for SDD (Table 4.3). This is an average decrease of 13.32% and 37.77% respectively for SD and SDD respectively. For pH C-SD a non-significant increase in antioxidant activity of 0.81-4.18% was measured. The measured decrease in activity following SD may have been due to the presence of pepsin. For pH C-SDD the measured change of antioxidant activity was not significant while in the presence of pancreatin, measured loss of antioxidant activity was 23.77-74.82%. For all samples pH C-SD > SD and pH C-SDD > SDD, indication that the binding of polyphenolics to protein reduces antioxidant activity. For unfermented green tea pH C-SD increased by 7.10% and for pH C-SDD increased by 6.91%. Antioxidant activity was decreased by 6.27% for SD and decreased by 55.97% for SDD. Changes in activity were biologically relevant for SD for most samples and for SDD, all samples.

To summarise the decrease in antioxidant activity due to SD and SDD was 13.32% and 37.77% respectively and this change in activity was not due to changes in pH (Table 4.5).

The ORAC assay measures antioxidant activity against biologically relevant free radicals. In this study indications are that with SD and SDD antioxidant activity is reduced but this was not due to the effects of pH. Polyphenols may interact with globular proteins and form non-covalent bonds that result in complexation (Chaudhuri, Chakraborty & Sengupta, 2011), stabilization of protein structure (Kanakis *et al*., 2011), and unfolding and precipitation of protein. Factors that influence the strength of interactions include the size and structure of polyphenols, as well as amino-acid sequence of proteins (Frazier *et al*., 2010). When polyphenols form complexes with proteins, the polyphenols' electron donating ability can be affected and the number of hydroxyl groups available in solution reduced, which in turn affects the antioxidant activity of the polyphenols (Arts *et al*., 2001).

4.4.3 Antioxidant activity of Aspalathus linearis: DPPH, TEAC and ORAC

The antioxidant activity of *Aspalathus linearis* tea was determined using DPPH, TEAC and ORAC assays. Antioxidant activity as well as the change in activity following stomach and duodenal digestion is presented in Table 4.4.

4.4.3.1 DPPH

Radical scavenging ability measured with the DPPH assay of fermented Rooibos tea was 39.86-52.82 mg TE/g for UD tea, which decreased with SD to 23.24-27.74 mg TE/g and then to 26.13-36.81 mg TE/g for SDD (Table 4.4). pH 2 alone caused a 72.45-84.51% decrease in antioxidant activity while pH 2 in the presence of pepsin resulted in a 50.06-65.59% decrease in activity. A change in pH to 7 following SD, caused a 70.47-84.20% decrease in antioxidant activity which, compared to digestion at pH 7 in the presence of pancreatin, was 35.72-41.61%. After SD the antioxidant activity increased with SDD. Unfermented Rooibos tea showed a slightly different trend to fermented Rooibos tea. Antioxidant activity for SDD of unfermented Rooibos tea was slightly lower than that for SD.

Viljoen (2008, pp.177-178) reported that aspalathin, orientin and iso-orientin content of fermented and unfermented Rooibos tea decreased significantly at pH 6 and 7, which is what was also observed in our study. At the lowest pH parameter in their study, pH 3, no significant decrease was observed, which is in contrast to the effect observed in this study at pH 2 where antioxidant activity was reduced.

Table 4.4: Antioxidant activity of undigested and adult digested fermented and unfermented *Aspalathus linearis* tea

Sample		DPPH		TEAC		ORAC	
		mg TE/g	% Change	mg TE/g	% Change	mg TE/g	% Change
Fermented							
F	UD	$39.86^{i} \pm 1.08$	–	$16.63^{ghi} \pm 1.09$	–	$555.37^{cde} \pm 24.95$	–
	SD	$23.90^{de} \pm 0.35$	↓ **50.06**	$10.06^{a} \pm 0.58$	↓ **49.23**	$478.65^{b} \pm 39.10$	↓ **14.84**
	pH C-SD	$18.66^{a} \pm 1.56$	↓ **72.45**	$14.11^{cdef} \pm 0.94$	↓ **16.40**	$586.57^{de} \pm 21.30$	↑ 5.46
	SDD	$26.13^{ef} \pm 2.55$	↓ **41.61**^{ns}	$13.99^{cdef} \pm 1.62$	↓ **17.24**^{s}	$370.20^{a} \pm 43.66$	↓ **40.01**^{s}
	pH C-SDD	$19.09^{ab} \pm 0.10$	↓ **70.47**	$15.01^{efg} \pm 1.30$	↓ **10.24**	$595.93^{e} \pm 8.85$	↑ 7.05
L	UD	$52.82^{k} \pm 2.43$	–	$20.88^{k} \pm 1.49$	–	$608.00^{e} \pm 34.12$	–
	SD	$27.74^{f} \pm 0.48$	↓ **62.26**	$15.80^{fg} \pm 1.14$	↓ **27.70**	$526.79^{bc} \pm 60.25$	↓ **14.31**
	pH C-SD	$21.44^{bc} \pm 1.28$	↓ **84.51**	$18.11^{hij} \pm 0.44$	↓ **14.21**	$603.50^{e} \pm 1.46$	↓ 0.74
	SDD	$36.81^{h} \pm 1.67$	↓ **35.72**^{s}	$19.57^{jk} \pm 1.80$	↓ 6.48^{s}	$398.91^{a} \pm 42.59$	↓ **41.53**^{s}
	pH C-SDD	$23.43^{cd} \pm 0.23$	↓ **77.09**	$14.75^{defg} \pm 1.23$	↓ **34.41**	$592.03^{e} \pm 3.04$	↓ 2.66
P	UD	$45.92^{j} \pm 1.39$	–	$19.63^{jk} \pm 1.19$	–	$573.70^{cde} \pm 21.46$	–
	SD	$23.24^{cd} \pm 0.58$	↓ **65.59**	$10.99^{ab} \pm 0.90$	↓ **56.43**	$533.77^{bcd} \pm 55.46$	↓ 7.21
	pH C-SD	$19.22^{ab} \pm 2.09$	↓ **81.98**	$16.04^{fg} \pm 1.56$	↓ **20.13**	$590.74^{e} \pm 10.86$	↑ 2.93
	SDD	$30.35^{g} \pm 1.78$	↓ **40.83**^{s}	$18.58^{ij} \pm 1.99$	↓ 5.50^{s}	$369.99^{a} \pm 41.58$	↓ **43.17**^{s}
	pH C-SDD	$18.71^{a} \pm 0.14$	↓ **84.20**	$12.49^{bc} \pm 0.55$	↓ **44.46**	$582.41^{cde} \pm 7.25$	↑ 1.51
Unfermented-Control							
L	UD	$64.30^{l} \pm 1.92$	–	$29.87^{l} \pm 1.27$	–	$600.98^{e} \pm 35.46$	–
	SD	$34.47^{h} \pm 0.65$	↓ **60.40**	$13.59^{cde} \pm 0.64$	↓ **74.92**	$531.11^{bcd} \pm 69.93$	↓ **12.34**
	pH C-SD	$25.37^{def} \pm 2.26$	↓ **86.83**	$19.20^{jk} \pm 1.47$	↓ **43.49**	$598.31^{e} \pm 5.54$	↓ 0.45
	SDD	$31.83^{g} \pm 1.61$	↓ **67.55**^{s}	$16.23^{gh} \pm 1.45$	↓ **59.18**^{s}	$362.72^{a} \pm 31.83$	↓ **49.45**^{s}
	pH C-SDD	$23.41^{cd} \pm 0.30$	↓ **93.24**	$12.79^{bcd} \pm 0.94$	↓ **80.08**	$609.56^{e} \pm 0.21$	↑ 1.42

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)
Antioxidant activity multiply by 4 to convert to mmol/L

Digests compared to pH controls was SD> pH C-SD and SDD > pH C-SDD which implies that the presence of the digestive enzymes protect against pH associated degradation. If the polyphenolics have bound the enzymes, the concentration of polyphenolics that can undergo pH driven oxidation is reduced. All changes in activity were biologically relevant.

In summary, antioxidant activity measured with the DPPH assay of digested Rooibos tea decreased by 59.15% for SD and then increased for SDD, with 67.39% activity remaining following SDD. In comparison the antioxidant activity of UD, SD and SDD black tea was significantly greater than the same fractions of fermented Rooibos tea (Table 4.5).

4.4.3.2 TEAC

The antioxidant activity of UD fermented Rooibos tea was 16.63-20.88 mg TE/g, which decreased to 10.06-15.80 mg TE/g following SD and following SDD to 13.99-19.57 mg TE/g (Table 4.4). This translates to an average decrease in antioxidant activity compared to UD tea of 45.16% and 11.11% for SD and SDD respectively, so antioxidant activity increased by 34.05% following SDD. For pH C-SD the loss of activity was 14.21-20.13% compared to 27.70-56.43% for SD. In contrast to the DPPH assay, pH C-SD > SD while the effect of SDD was similar with SDD > pH C-SDD. This means that in the presence of the digestive enzymes antioxidant activity is reduced. For unfermented Rooibos tea a 74.92% and 59.18% decrease in antioxidant activity was measured for SD and SDD respectively.

In summary for fermented Rooibos tea SD caused a 45.16% decrease in antioxidant activity while for SDD antioxidant activity increased and was not significantly different from undigested tea (Table 4.5).

Viljoen (2008, pp.177-178) observed that in an acidic environment the antioxidant activity of aspalathin, orientin and iso-orientin remained unchanged while at a neutral pH activity was reduced. In this study the antioxidant activity measured using the TEAC assay of Rooibos tea was reduced with the effect of SD being greater than pH C-SD. The opposite occurred with SDD, where pH effects were responsible for the decrease in antioxidant activity while the presence of pancreatin resulted in some protection from pH induced polyphenol degradation or release of polyphenolics (Cilla *et al*., 2011).

In comparison to UD, SD and SDD Rooibos tea, black tea has greater antioxidant activity measured with the TEAC assay than Rooibos tea.

4.4.3.3 ORAC

The antioxidant activity measured with the ORAC assay for undigested fermented Rooibos tea was 555.37-608.00 mg TE/g which decreased significantly following SD to 478.65-533.77 mg TE/g and then to 369.99-398.91 mg TE/g following SDD (Table 4.4). Antioxidant activity following SD was 7.21-14.84% less than UD tea. The effect of pH C-SD was not significant compared to UD but differences between pH C-SD and SD was significant. Therefore the inclusion of pepsin at pH 2 may have caused a loss of antioxidant activity. Following SDD antioxidant activity measured with the ORAC assay was 40.01-43.17% less than UD tea and was biologically relevant for all samples. pH C-SD > SD and pH C-SDD > SDD and reflects the findings of the DPPH assay.

In summary SD and SDD resulted in a 12.09% and 41.50% decrease respectively in antioxidant activity. The same trend that was observed with black and green tea was observed for fermented and unfermented Rooibos tea.

The effects of digestion on antioxidant content and activity expressed as activity per ml are presented in Table 4.5. Digestion caused an increase in TPC with SD which decreased with SDD, meaning that digestion had no effect on the TPC of Rooibos tea. For black tea with digestion TFC levels were unchanged while for Rooibos tea, TFC levels were reduced. For black tea 75.00% and 97.78% of TPC and TFC were retained respectively. For Rooibos tea 100% and 89.83% of TPC and TFC respectively were retained. This indicates that with digestion a large fraction of the polyphenolics are still present. In general for both types of tea digestion caused a decrease in measured SD antioxidant activity of 13.32-31.19% for black tea and 12.09-59.15% for Rooibos tea, which was dependent on the type of tea and principles of the assays used.

With further digestion black tea antioxidant activity was reduced by 62.50% and 37.77% when measured with the DPPH and ORAC assays respectively. The remaining measured activity was 52.38%, 94.64% and 68.23% for the DPPH, TEAC and ORAC assays respectively. Digestion of Rooibos tea resulted in a 11.11-41.50% loss in antioxidant activity with the amount of activity remaining after digestion being 67.39%, 89.47% and 65.63% measured with the DPPH, TEAC and ORAC assays respectively. These results clearly show that although digestion does affect antioxidant activity a significant portion of initial activity is retained, the measured activity of black tea was greater than Rooibos tea, and the presence of matrix such as proteolytic enzymes and/or pH can modulate activity i.e. cause a decrease or increase in antioxidant activity.

Table 4.5: Summary of antioxidant properties of undigested and adult digested fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea per ml

Sample	TPC		TFC		DPPH		TEAC		ORAC	
	mg GAE/ml	% Change	mg CE/ml	% Change	mg TE/ml	% Change	mg TE/ml	% Change	mg TE/ml	% Change
Black Tea										
UD	$0.28^{c} \pm 0.02$	–	$0.90^{a} \pm 0.12$	–	$1.26^{e} \pm 0.04$	–	$0.56^{b} \pm 0.08$	–	$11.93^{e} \pm 0.24$	–
SD	$0.33^{d} \pm 0.05$	↑ **16.39**	$0.88^{a} \pm 0.14$	↓ 2.25	$0.92^{d} \pm 0.07$	↓ **31.19**	$0.44^{d} \pm 0.10$	↓ **24.00**	$10.44^{d} \pm 0.43$	↓ **13.32**
SDD	$0.21^{b} \pm 0.04$	↓ **28.57**^{s}	$0.88^{a} \pm 0.16$	↓ 2.25^{ns}	$0.66^{c} \pm 0.18$	↓ **62.50**^{s}	$0.53^{b} \pm 0.13$	↓ 5.50^{ns}	$8.14^{c} \pm 1.81$	↓ **37.77**^{s}
% Remaining content/activity	**75.00%**		**97.78%**		**52.38%**		**94.64%**		**68.23%**	
Fermented Rooibos Tea										
UD	$0.09^{a} \pm 0.01$	–	$0.59^{c} \pm 0.10$	–	$0.46^{b} \pm 0.06$	–	$0.19^{a} \pm 0.02$	–	$5.79^{a} \pm 0.27$	–
SD	$0.10^{a} \pm 0.01$	↑ **10.53**	$0.48^{b} \pm 0.05$	↓ **20.56**	$0.25^{a} \pm 0.02$	↓ **59.15**	$0.12^{c} \pm 0.03$	↓ **45.16**	$5.13^{a} \pm 0.30$	↓ **12.09**
SDD	$0.09^{a} \pm 0.01$	0^{ns}	$0.53^{bc} \pm 0.07$	↓ **10.71**^{ns}	$0.31^{a} \pm 0.05$	↓ **38.96**^{ns}	$0.17^{a} \pm 0.03$	↓ **11.11**^{s}	$3.80^{b} \pm 0.17$	↓ **41.50**^{s}
% Remaining content/activity	**100%**		**89.83%**		**67.39%**		**89.47%**		**65.63%**	

Data is an average of each tea type ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)

***Bold** indicates biological relevant difference from UD (≥ 10% in % Change)*

$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

Antioxidant activity multiply by 4 to convert to mmol/L

4.4.4 Cellular and biological assays

4.4.4.1 NO scavenging ability of Camellia sinensis

UD black tea had the highest NO scavenging ability of 70.38-73.62% compared to Rooibos tea. Scavenging ability of black tea increased with SD, although not statistically significant, to 71.73-77.06 % and decreased to 52.55-67.13% for SDD (Table 4.6). For pH C-SD and pH C-SDD the scavenging activity was 60.51-68.06% and 31.46-60.44% respectively. The NO scavenging activity was greater for SD compared to pH C-SD, and for SDD compared to pH C-SDD, which indicates as for antioxidant activity the presence of protein may prevent pH driven degradation of polyphenolics or that the added protein (i.e. enzymes) also had inherent NO scavenging ability. Biologically relevant decrease in NO scavenging ability was found for SDD of all samples except P. Activity retained following SDD was high and was 52.55-67.13%. Average remaining NO scavenging activity was 81.34% of UD (Table 4.10).

For green tea the NO scavenging activity of SD, 71.41% was significantly greater than UD, 66.39% which with SDD decreased significantly to 61.22%. As for black tea, significant differences in activity of green tea were found between pH C-SD and SD as well as pH C-SDD and SDD.

In general (Table 4.10), with digestion the NO scavenging activity of black tea expressed as mg TE per ml for SD remains unchanged but is reduced by 20.58% with SDD when compared to UD.

NO scavenging ability of black tea had a similar trend to that of TFC of black tea. During stomach digestion of black tea, it is possible that polyphenolic compounds were either protected by enzymes or released and broken down by enzymes (Cilla *et al*., 2011) to products that were more stable at acidic pH, and therefore NO scavenging ability remained stable. The decreased in NO scavenging ability of black tea with pH C-SD, SDD, and pH C-SDD, may be as a result of polyphenolic compounds present that were less stable at acidic and/or neutral pH, leading to oxidation and subsequent degradation of these compounds (Record & Lane, 2001).

The increase in NO scavenging ability of green tea from undigested to SD could be as a result of enzymes binding to polyphenolic compounds, breaking them down and releasing them (Cilla *et al*., 2011), thereby increasing NO scavenging ability as well. NO scavenging ability remained stable during pH C-SD of green tea, which could indicate that green tea

polyphenolic compounds are stable at acidic pH (Neilson *et al*., 2007). The decrease observed with SDD and pH C-SDD is most likely due to oxidation and degradation of phenolic compounds (Neilson *et al*., 2007), resulting in lowering of the NO scavenging ability of green tea.

Table 4.6: NO scavenging ability of undigested and adult digested fermented and unfermented *Camellia sinensis* tea

Sample		NO Scavenged		
		% Scavenged	TE (µmol/L)	% Change
Fermented				
W	UD	$70.38^{hij} \pm 2.53$	773.86	–
	SD	$71.73^{j} \pm 3.55$	788.71	↑ 1.90
	pH C-SD	$60.51^{d} \pm 0.43$	665.36	↓ **15.08**
	SDD	$54.40^{c} \pm 0.67$	598.22	↓ **25.60**^{s}
	pH C-SDD	$34.65^{ab} \pm 0.10$	381.00	↓ **68.04**
G	UD	$71.19^{ij} \pm 1.30$	782.87	–
	SD	$72.54^{jk} \pm 3.54$	797.63	↑ 1.87
	pH C-SD	$63.43^{def} \pm 0.77$	697.53	↓ **11.53**
	SDD	$52.55^{c} \pm 2.17$	577.83	↓ **30.14**^{s}
	pH C-SDD	$34.69^{ab} \pm 0.95$	381.47	↓ **68.95**
F	UD	$73.12^{jk} \pm 2.28$	804.03	–
	SD	$76.02^{kl} \pm 4.06$	835.89	↑ 3.89
	pH C-SD	$65.32^{fg} \pm 0.26$	718.30	↓ **11.26**
	SDD	$60.39^{d} \pm 1.54$	664.09	↓ **19.06**^{s}
	pH C-SDD	$31.46^{a} \pm 1.91$	345.98	↓ **79.66**
P	UD	$73.62^{jkl} \pm 1.36$	809.51	–
	SD	$77.06^{l} \pm 2.59$	847.42	↑ 4.58
	pH C-SD	$68.06^{ghi} \pm 0.94$	748.34	↓ 7.85
	SDD	$67.13^{gh} \pm 1.65$	738.13	↓ 9.22^{s}
	pH C-SDD	$60.44^{d} \pm 0.59$	664.59	↓ **19.66**
Unfermented-Control				
W	UD	$66.39^{fg} \pm 4.43$	730.03	–
	SD	$71.41^{ij} \pm 2.68$	785.20	↑ 7.28
	pH C-SD	$64.72^{efg} \pm 1.17$	711.71	↓ 2.54
	SDD	$61.22^{de} \pm 2.34$	673.13	↓ 8.11^{s}
	pH C-SDD	$35.99^{b} \pm 2.39$	395.71	↓ **59.40**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

Table 4.7: NO scavenging ability of undigested and adult digested fermented and unfermented *Aspalathus linearis* tea

Sample		NO Scavenged		
		% Scavenged	**TE (µM)**	**% Change**
Fermented				
F	UD	$34.46^{fg} \pm 3.91$	378.92	–
	SD	$26.92^{bc} \pm 3.38$	295.99	↓ **24.58**
	pH C-SD	$29.45^{cde} \pm 1.22$	323.78	↓ **15.69**
	SDD	$20.59^{a} \pm 1.47$	226.43	↓ **50.38**^{s}
	pH C-SDD	$28.43^{bcd} \pm 1.11$	312.59	↓ **19.18**
L	UD	$37.80^{gh} \pm 4.18$	415.67	–
	SD	$33.43^{efg} \pm 3.73$	367.56	↓ **12.29**
	pH C-SD	$30.07^{cdef} \pm 1.71$	330.68	↓ **22.77**
	SDD	$25.89^{bc} \pm 1.64$	284.66	↓ **37.41**^{s}
	pH C-SDD	$29.52^{cde} \pm 0.93$	324.64	↓ **24.59**
P	UD	$37.85^{gh} \pm 4.18$	416.21	–
	SD	$34.18^{fg} \pm 2.22$	375.87	↓ **10.19**
	pH C-SD	$32.02^{def} \pm 2.24$	352.13	↓ **16.68**
	SDD	$25.79^{bc} \pm 1.51$	283.58	↓ **37.91**^{s}
	pH C-SDD	$26.13^{bc} \pm 1.01$	287.30	↓ **36.65**
Unfermented-Control				
L	UD	$44.58^{i} \pm 5.21$	490.18	–
	SD	$46.61^{i} \pm 4.04$	512.49	↑ 4.45
	pH C-SD	$39.02^{h} \pm 1.89$	429.10	↓ **13.29**
	SDD	$28.17^{bcd} \pm 1.41$	309.76	↓ **45.11**^{s}
	pH C-SDD	$24.34^{ab} \pm 1.65$	267.64	↓ **58.73**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

4.4.4.2 NO scavenging ability of Aspalathus linearis

The NO scavenging ability of UD Rooibos tea was 34.46-37.85% which with digestion decreased to 26.92-34.18%, which was only statistically significant for F (Table 4.7). Differences between SD and pH C-SD were not statistically significant. With SDD, NO scavenging activity was 20.59-25.89% and was statistically less than UD and SD. Changes in NO scavenging ability was biologically relevant. The average remaining activity was 65.59% of UD (Table 4.10). Unfermented Rooibos tea had higher values than fermented Rooibos tea for most treatments. The trend of change of NO scavenging activity is similar to the measured

TFC, indicating possibly that the flavanoids in Rooibos tea that undergo pH dependent degradation (Viljoen, 2008, pp. 177-178) also play a role in NO scavenging.

As summarised in Table 4.10, with digestion the NO scavenging activity of Rooibos tea expressed as mg TE per ml decreases by 15.47% and 41.55% for SD and SDD respectively, compared to UD tea. Both types of tea are able to scavenge NO thereby preventing the formation of RNS. Black tea compared to Rooibos tea has higher NO scavenging ability in all stages of digestion.

4.4.4.3 DCFH-DA of Camellia sinensis

The ability of each tea digest to protect against oxidation was evaluated using the DCHF-DA assay in the Caco-2 cell line. The antioxidant mechanisms involved are either a direct scavenging effect usually within the extracellular environment and/or indirectly by preventing intercellular ROS generation (Yoo *et al*., 2008). All tea digests were evaluated for oxidative/cytotoxic effects following exposure of the Caco-2 cells to the digests in the absence of AAPH. All samples did not cause ROS generation (data not shown).

AAPH alone caused 100% cellular damage and with the inclusion of polyphenol rich extracts a decrease in the % damage was observed. All digests protected the Caco-2 cells against AAPH induced oxidative damage. An increase in % damage implies a loss in the cellular antioxidant activity of the digests. The percentage damage of UD black tea was 2.47-3.75% compared to 100% for AAPH. This increased to 4.63-7.57% for SD and was significantly less than pH C-SD. For SDD the % damage increased to 11.68-23.57% and was significantly greater than pH C-SDD. For unfermented control green tea the % damage was -5.5 ± 0.62% which indicates a possible pro-oxidant effect (Tao *et al*., 2014) i.e. in the presence of AAPH there is an increase in cellular damage.

To summarise with digestion the % damage was 6.10% and 16.85% for SD and SDD respectively. Compared to SD, pH C-SD was significantly higher while SDD compared to pH C-SDD was lower.

No literature was found related to the cellular antioxidant activity of either black or Rooibos tea. According to Feng *et al*. (2002), antioxidant/pro-oxidant activity of tea as seen in this research is due to a complex mixture of polyphenols that have metal-reducing activity, chelating behaviour, variable solubility characteristics, bioavailability, and stability in tissues and cells.

Table 4.8: Cellular protective effect of undigested and adult digested fermented and unfermented *Camellia sinensis* tea

Sample		DCFH-DA	
		% Damage	% Change
Fermented			
W	UD	$3.55^{bcd} \pm 0.14$	–
	SD	$6.41^{fgh} \pm 0.34$	↑ **57.43**
	pH C-SD	$7.85^{ij} \pm 0.82$	↑ **75.44**
	SDD	$15.52^{m} \pm 1.12$	↑ **125.44**^{s}
	pH C-SDD	$8.22^{ij} \pm 0.57$	↑ **79.35**
G	UD	$3.33^{bcd} \pm 0.30$	–
	SD	$5.79^{ef} \pm 0.51$	↑ **53.95**
	pH C-SD	$7.15^{ghi} \pm 0.60$	↑ **72.90**
	SDD	$23.57^{n} \pm 2.52$	↑ **150.48**^{s}
	pH C-SDD	$7.33^{ghi} \pm 0.66$	↑ **75.05**
F	UD	$2.47^{b} \pm 0.22$	–
	SD	$4.63^{de} \pm 0.48$	↑ **60.85**
	pH C-SD	$7.18^{ghi} \pm 0.45$	↑ **97.62**
	SDD	$16.64^{m} \pm 0.83$	↑ **148.30**^{s}
	pH C-SDD	$3.24^{bc} \pm 0.14$	↑ **26.97**
P	UD	$3.75^{bcd} \pm 0.18$	–
	SD	$7.57^{hi} \pm 0.74$	↑ **67.49**
	pH C-SD	$11.07^{k} \pm 1.05$	↑ **98.79**
	SDD	$11.68^{kl} \pm 0.77$	↑ **102.79**^{s}
	pH C-SDD	$3.71^{bcd} \pm 0.32$	↓ 1.07
Unfermented-Control			
W	UD	$-5.57^{a} \pm 0.62$	–
	SD	$6.09^{fg} \pm 0.73$	*
	pH C-SD	$9.10^{j} \pm 0.59$	*
	SDD	$12.96^{l} \pm 1.21$	*
	pH C-SDD	$4.48^{cde} \pm 0.53$	*

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
** Cannot be determined due to undigested value being negative, hence having a pro-oxidant effect*
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

In *in vitro* digestion and cell culture media, a by-product of catechin auto-oxidation is known to be catechin auto-oxidation dimers that include theasinensins, which may have entirely different bioactivities than their native monomeric catechins (Ferruzzi, 2010). Tea digests can be considered as complex mixtures of newly formed polyphenolic derivatives, degraded and

oxidised polyphenolic products. In addition the presence of matrix elements such as proteolytic enzymes may result in ROS quenching or polyphenol binding.

4.4.4.4 DCFH-DA of Aspalathus linearis

The ability of Rooibos extracts to protect Caco-2 cells against AAPH induced oxidative damage was also determined. AAPH alone caused 100% damage while 14.67-16.64% damage was observed with UD Rooibos tea (Table 4.9). SD caused no significant change in the % damage measured while for pH C-SD a significant decrease in % damage was found for pH C-SD compared to SD (10.60-14.02% vs. 13.41-17.75%). For SDD the % damage increased to 27.46-31.35% which was greater than the 17.23-26.63% damage found for pH C-SDD. In general with SD cellular antioxidant activity was similar to UD, while with SDD activity decreased by 49.07-72.49%. This data reflects the findings of Viljoen (2008, pp. 177-178) where Rooibos flavonoids were stable at low pH and degraded with loss of activity at neutral pH. A combination of mild temperature and low pH could induce changes in the fermented Rooibos tea matrix, leading to an increased availability of aspalathin. These conditions could release aspalathin from a loose association with polymeric phenolic compounds, matrix proteins or carbohydrates. In the present study conditions such as low pH in the stomach and incubation at 37°C did not result in increased cellular protection.

Unfermented Rooibos tea pH C-SDD caused the lowest oxidative damage (8.28%). There was a slight but not statistically significant decrease in % damage from undigested (13.16%) to SD (11.69%) unfermented Rooibos tea. A very slight increase was observed with pH C-SD (15.17%). With SDD, a significant increase was found in % damage of unfermented Rooibos tea (19.16%. A significant increase in protection was seen with pH C-SDD (91.72%).

The cellular protection of SDD of both fermented and unfermented Rooibos tea, as well as fermented Rooibos tea's pH C-SDD, decreased and this may be due to the findings of Viljoen (2008, pp. 177-178) that showed that aspalathin, iso-orientin and orientin content of fermented and unfermented Rooibos tea decreased significantly at neutral pH. Interestingly during pH C-SDD of unfermented Rooibos tea, the measured percentage damage was reduced. Protein is known to scavenge ROS and this binding may account for the decrease in measured activity found for SDD.

In summary, cellular protection of fermented Rooibos tea expressed as % protection decreased by 0.32% following SD and 16.55% following SDD, with 71.71% protection remaining after SDD (Table 4.10).

Black tea compared to Rooibos tea, data expressed as % protection shows that black tea better protects Caco-2 cells against oxidative damage (83.15% vs. 71.71%). For both tea types SDD caused a significant decrease in cellular antioxidant activity.

Table 4.9: Cellular protective effect of undigested and adult digested fermented and unfermented *Aspalathus linearis* tea

Sample		DCFH-DA	
		% Damage	**% Change**
Fermented			
F	UD	$16.64^{efg} \pm 1.54$	–
	SD	$17.75^{fgh} \pm 1.86$	↑ 6.46
	pH C-SD	$14.02^{cd} \pm 1.11$	↓ **17.09**
	SDD	$27.46^{i} \pm 1.78$	↑ **49.07**^{s}
	pH C-SDD	$26.63^{i} \pm 3.83$	↑ **46.18**
L	UD	$14.74^{de} \pm 1.12$	–
	SD	$13.41^{cd} \pm 0.63$	↓ 9.45
	pH C-SD	$10.60^{ab} \pm 1.27$	↓ **32.68**
	SDD	$26.06^{i} \pm 1.58$	↑ **55.49**^{s}
	pH C-SDD	$17.23^{efgh} \pm 1.28$	↑ **15.58**
P	UD	$14.67^{de} \pm 1.07$	–
	SD	$15.69^{def} \pm 0.38$	↑ 6.72
	pH C-SD	$10.70^{ab} \pm 1.14$	↓ **31.30**
	SDD	$31.35^{j} \pm 2.58$	↑ **72.49**^{s}
	pH C-SDD	$19.52^{h} \pm 1.44$	↑ **28.37**
Unfermented-Control			
L	UD	$13.16^{bcd} \pm 0.86$	–
	SD	$11.69^{bc} \pm 0.95$	↓ **11.83**
	pH C-SD	$15.17^{de} \pm 0.78$	↑ **14.19**
	SDD	$19.16^{gh} \pm 1.37$	↑ **37.13**^{s}
	pH C-SDD	$8.28^{a} \pm 1.37$	↓ **45.52**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

Table 4.10: Summary of NO scavenging properties and cellular protective effects of fermented *Camellia sinensis* and *Aspalathus linearis* tea per ml following adult digestion

Sample	NO Scavenging		DCFH-DA	
	mg TE/ml	% Change	% Protection	% Change
Black Tea				
UD	7. 93^{a} ± 0.08	–	96.72^{e} ± 0.05	–
SD	8.17^{a} ± 0.04	↑ 2.98	93.90^{d} ± 0.31	↓ 2.96
SDD	6.45^{e} ± 0.10	↓ **20.58**^{s}	83.15^{a} ± 0.70	↓ **15.09**^{s}
% Remaining activity	**81.34%**		**83.15%**	
Rooibos Tea				
UD	4.04^{d} ± 0.21	–	84.65^{b} ± 0.81	–
SD	3.46^{c} ± 0.44	↓ **15.47**	84.38^{ab} ± 0.73	↓ 0.32
SDD	2.65^{b} ± 0.33	↓ **41.55**^{s}	71.71^{c} ± 1.29	↓ **16.55**^{s}
% Remaining activity	**65.59%**		**71.71%**	

Data is an average of each tea type ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change)*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

4.5 Conclusion

For black tea, SD caused an increase in the extraction of polyphenolics which with further digestion decreased to 28.57% less than UD. No changes in TFC levels were found with digestion. Antioxidant activity of digested black tea measured with the DPPH assay decreased by 31.19% for SD and for SDD 62.50% compared to UD. Remaining activity following digestion was 52.38%. With the TEAC assay the measured antioxidant activity decreased by 24.00% for SD and for SDD was unchanged compared to UD. Following SDD the remaining antioxidant activity was 94.64% of UD. Further evaluation of antioxidant activity with the ORAC assay showed that with SD and SDD activity was reduced by 13.32% and 37.77% compared to UD, respectively. Remaining activity following SDD was 68.23%. NO scavenging activity of black tea was unchanged for SD but was reduced by 20.58% for SDD compared to UD. Evaluation of cellular antioxidant activity revealed that SD and SDD caused a loss of 2.96% and 15.09% respectively in cellular protection and the remaining activity was 83.15%.

For fermented Rooibos tea, SD increased by 10.53% TPC levels, while further digestion resulted in no significant difference in TPC compared to UD tea. In contrast SD and SDD resulted in a 20.56% and a 10.71% decrease in TFC. Antioxidant activity of digested Rooibos tea measured with the DPPH assay decreased by 59.21% for SD and 38.96% for SDD compared to UD. Remaining activity following digestion was 67.39%. With the TEAC assay antioxidant activity with SD and SDD decreased by 45.16% and 11.11% respectively,

compared to UD tea, while 89.47% activity remained after digestion. The ORAC assay revealed that antioxidant activity was reduced by 12.09% and 41.50% for SD and SDD respectively while the remaining activity compared to UD was 65.63%. NO scavenging activity of Rooibos tea decreased 15.47% and 41.55% for SD and SDD respectively. Cellular antioxidant activity was decreased by 0.32% and 16.55% for SD and SDD respectively with the remaining activity being 71.71%.

Interestingly, the presence of proteins such as the digestive enzymes seems to modulate measured antioxidant activity either by possibly quenching free radicals or by directly binding polyphenols.

Black tea and Rooibos tea are both rich in polyphenolics although the composition and structure differ greatly. Antioxidant activity gave different results however in general SD caused a decrease in antioxidant activity which decreased further with SDD. Black tea consistently showed a better activity than Rooibos tea, however both retain antioxidant activity and NO scavenging ability which results in cellular protection against oxidants. In conclusion, although adult digestion altered the parameters measured, *Camellia sinensis* and *Aspalathus linearis* teas still retain a significant amount of antioxidant activity to contribute to the antioxidant status.

Chapter 5: Effect of simulated infant GIT digestion on the antioxidant properties of *Camellia sinensis* and *Aspalathus linearis* teas

5.1 Abstract

Tea is sometimes given to infants as early as 6 months of age and is a cost effective way of increasing antioxidant intake, and plays a role in modulation of the immune system and the inflammatory response. The antioxidant properties of commercially available *Camellia sinensis* and *Aspalathus linearis* teas and their infant *in vitro* digests were determined, and their ability to protect against oxidative damage in a cellular environment. TPC and TFC was determined by Folin-Ciocalteu and aluminium chloride assays, respectively, whereas antioxidant activity was determined by DPPH radical scavenging, TEAC and ORAC assays. Oxidative damage was determined by Caco-2 cell models and NO scavenging assay. *Camellia sinensis* and *Aspalathus linearis* teas increased in TPC and DPPH assays following SD and decreased following SDD. ORAC results of *Camellia sinensis* and *Aspalathus linearis* teas decreased in antioxidant activity with SD and SDD. TFC of black and Rooibos teas remained stable with SD and SDD. TEAC of black and fermented Rooibos teas was not significantly different from undigested tea following SDD. NO scavenging ability of *Camellia sinensis* and *Aspalathus linearis* teas decreased with all treatments. In the DCFH-DA assay, black tea and fermented Rooibos tea did not change significantly in % protection following digestion. Polyphenolic compounds may be oxidised and degraded by pH conditions of the stomach and small intestine. Acidic and alkaline hydrolysis of polyphenolic compounds may increase antioxidant content and activity. *Camellia sinensis* and *Aspalathus linearis* teas retained a substantial amount of antioxidant activity after infant digestion, demonstrating that these teas are affordable sources of antioxidants for infants when consumed as part of a healthy, balanced diet.

5.2 Introduction

In South Africa, tea is widely consumed by all communities. Black tea is more commonly consumed in the North-West province, while Rooibos tea is typically consumed in the Western Cape (Breedt *et al.*, 2005). Tea is often introduced into the diet at as early as 6 months of age (Faber & Benadé, 2007; Mushaphi *et al.*, 2008). Tea and tea containing beverages are also increasingly being marketed at infants.

Faber (2004) found that 18% of infants aged 6-12 months from rural South African communities were beta-carotene/vitamin A deficient. The National Food Consumption Survey (Labadarios *et al*., 2005) found that vitamin A deficiency is a serious concern in South Africa and as result the antioxidant status of this population would also be low. Although it cannot address basic vitamin deficiencies, tea consumption is a cost effective way of increasing antioxidant intake, and plays a role in modulation of the immune system and the inflammatory response (Serafini, Peluso & Raguzzini, 2010).

Infant digestion differs from adult digestion, in that during stomach digestion in infants, pH is roughly 4 as opposed to pH 2 in adult stomach digestion (Li-Chan & Nakai, 1989). The stability of polyphenolics is dependent on the pH of the environment, where at a low pH polyphenols are stable (Record & Lane, 2001) but undergo auto-degradation at a neutral pH (Ferruzzi, 2010; Spencer, 2003; Green *et al*., 2007; Viljoen, 2008). Some studies have also found that acidic conditions have no significant effect on antioxidant content and activity of tea (Viljoen, 2008; Neilson *et al*., 2007) however little is known about the effect of pH 4 and the effect of further digestion on the antioxidant properties of tea. As found in Chapter 4 the presence of a matrix such as protein can also alter activity therefore the concentration of enzymes were kept the same as for adult digestion although other studies make use of lower concentrations of digestive enzymes (Miquel *et al*., 2005).This will also allow the evaluation of the effect of pH alone.

In this study the effect of infant digestion, specifically related to pH, on the antioxidant content and activity, NO scavanging ability as well as cellular antioxidant activity *Camellia sinensis* and *Aspalathus linearis* was determined.

5.3 Materials and Methods

5.3.1 In vitro simulated infant gastrointestinal digestion

Tea samples used in this study were the same as those used in Chapters 3 and 4. Tea extracts were prepared as described in Chapter 3, section 3.3. Samples were then digested using an *in vitro* digestion model that simulates infant digestion (Figure 5.1). Samples were adjusted to pH 4 with 1 M HCl, 5 µl pepsin (20 mg/ml) per ml of sample added to each sample (except pH controls) and incubated for 30 min in 37°C water bath to simulate stomach digestion (SD). Stomach control pH 4 (pH C-SD) and SD tea samples were removed. The remaining samples were adjusted to pH 7 with 1 M $NaHCO_3$, 5 µl pancreatin (4 mg/ml) per ml of

sample added (except pH control) and incubated for 60 min in 37°C water bath to simulate duodenal digestion. This was known as stomach and duodenal digestion (SDD). Duodenal control pH 7 (pH C-SDD) and SDD tea samples were then removed. After removing stomach and duodenal digested tea samples, they were boiled for 5 min at 95°C to stop enzyme reaction and stored at -20°C until needed. All conditions and assays to measure antioxidant content and activity, as well as NO scavenging ability and cellular protective effects were the same as described in Chapter 3.

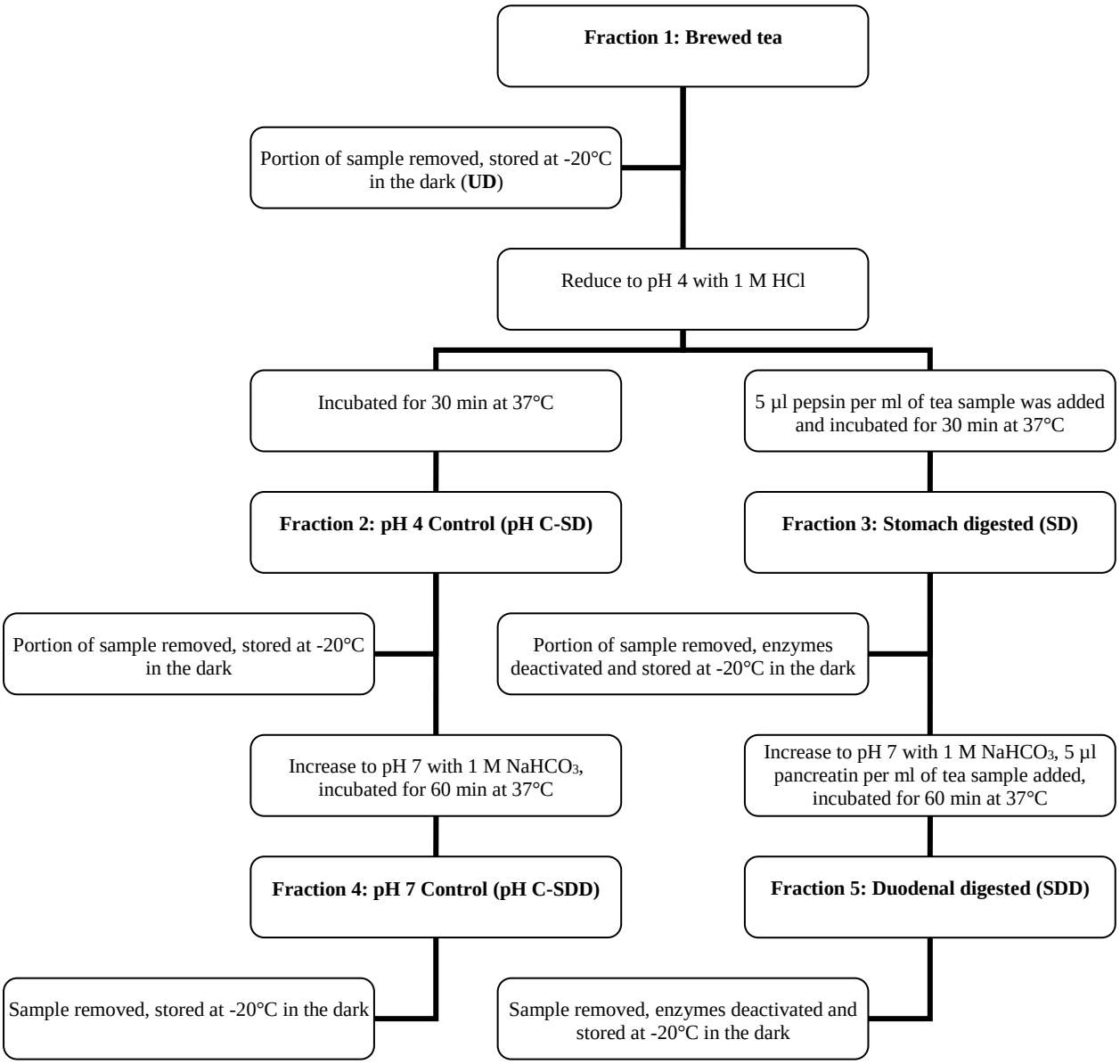

Figure 5.1: Methodology flow-chart of *in vitro* infant simulated gastrointestinal digestion

5.3.2 Statistical analysis

The means were subject to one-way ANOVA, calculated at the 5% level using STATISTICA Version 8 to compare treatment means for antioxidant content (TPC and TFC assays), antioxidant activity (DPPH, TEAC and ORAC assays), NO scavenging ability and cellular protective effects (DCFH-DA assay). One-way ANOVA was used when more than two sample means were compared.

% Change was calculated the following way:

$$\frac{\textbf{Undigested – Treatment (e.g. digest or control)}}{\textbf{((Undigested + Treatment) ÷ 2)}} \times \textbf{100 = \% Change}$$

5.4 Results & Discussion

A laboratory based model was used to determine the effect of infant digestion on the polyphenolic and flavonoid content of tea. The DPPH, TEAC and ORAC assays were used to determine the effect of digestion on antioxidant activity. The effect of digestion on the NO scavenging and cellular protective effects of the tea digests was also determined.

5.4.1 Antioxidant content

5.4.1.1 TPC and TFC – Camellia sinensis

The effect of infant digestion on TPC and TFC of black tea was determined (Table 5.1). UD black tea varied in TPC from 24.56-33.00 mg GAE/g. TPC increased with SDD (29.02-40.52 mg GAE/g). A significant decrease in TPC of black tea was found with pH C-SD (19.18-28.86 mg GAE/g) compared to UD. Differences between SD and pH C-SD were significant with SD > pH C-SD, which indicates an increased release of polyphenolics from the matrix. For SDD, TPC was 17.19-23.42 mg GAE/g which was significantly less for all samples than UD. For pH C-SDD, TPC levels were 13.76-21.14 mg GAE/g, indicating that the proteolytic digestive enzymes protect polyphenols against degradation.

TPC of green tea remained stable from its undigested state (27.86 mg GAE/g) to SD (27.58 mg GAE/g), although the pH C-SD (18.36 mg GAE/g) was significantly less. pH C-SDD (11.94 mg GAE/g) had the greatest impact of TPC levels causing a considerable loss in TPC when compared to SDD (20.12 mg GAE/g).

In summary, Table 5.5 shows that compared to undigested black tea, a significant increase of 16.39% in TPC was found with stomach digestion and a significant decrease of 33.33% was

observed with SDD. For adult digestion of black tea TPC was 16.39% and SDD was 28.57%. SD did effect TPC however with SDD, TPC was significantly less following infant digestion although a difference of 4.76% cannot be considered to be biologically relevant.

Table 5.1: Antioxidant content of undigested and infant digested fermented and unfermented *Camellia sinensis* tea

Sample		Total Polyphenolic Content		Total Flavonoid Content	
		mg GAE/g	% Change	mg CE/g	% Change
Fermented					
W	UD	$24.56^{ij} \pm 2.09$	–	$75.05^{cd} \pm 2.61$	–
	SD	$29.02^{m} \pm 0.85$	↑ **16.65**	$77.16^{cdef} \pm 1.44$	↑ 2.77
	pH C-SD	$19.18^{efg} \pm 0.60$	↓ **24.60**	$89.17^{hi} \pm 5.01$	↑ **17.20**
	SDD	$17.19^{cd} \pm 0.36$	↓ **35.31**^{s}	$77.45^{def} \pm 4.23$	↑ 3.15^{ns}
	pH C-SDD	$13.76^{b} \pm 0.61$	↓ **56.37**	$67.14^{a} \pm 3.37$	↓ **11.13**
G	UD	$26.85^{kl} \pm 1.40$	–	$81.76^{efg} \pm 3.00$	–
	SD	$30.95^{n} \pm 1.62$	↑ **14.19**	$86.15^{gh} \pm 1.42$	↑ 5.23
	pH C-SD	$20.32^{gh} \pm 0.85$	↓ **27.69**	$89.34^{hi} \pm 2.40$	↑ 8.86
	SDD	$18.19^{de} \pm 0.61$	↓ **38.45**^{s}	$82.79^{fg} \pm 4.17$	↑ 1.25^{ns}
	pH C-SDD	$15.77^{c} \pm 0.86$	↓ **51.99**	$99.68^{kl} \pm 1.31$	↑ **19.75**
F	UD	$28.50^{lm} \pm 1.90$	–	$99.09^{jkl} \pm 1.21$	–
	SD	$33.50^{o} \pm 1.84$	↑ **16.13**	$95.23^{jk} \pm 2.35$	↓ 3.97
	pH C-SD	$26.10^{jk} \pm 0.98$	↓ 8.79	$115.95^{m} \pm 6.21$	↑ **15.68**
	SDD	$20.04^{fgh} \pm 0.68$	↓ **34.86**^{s}	$93.81^{ij} \pm 4.07$	↓ 5.47^{ns}
	pH C-SDD	$17.00^{cd} \pm 0.89$	↓ **50.55**	$112.74^{m} \pm 1.24$	↑ **12.89**
P	UD	$33.00^{o} \pm 1.07$	–	$103.66^{l} \pm 1.50$	–
	SD	$40.52^{p} \pm 1.41$	↑ **20.46**	$116.34^{m} \pm 1.61$	↑ **11.53**
	pH C-SD	$28.86^{m} \pm 0.65$	↓ **13.39**	$127.17^{n} \pm 6.03$	↑ **20.37**
	SDD	$23.42^{i} \pm 0.76$	↓ **33.96**^{s}	$111.41^{m} \pm 5.93$	↑ 7.21^{ns}
	pH C-SDD	$21.14^{h} \pm 0.24$	↓ **43.81**	$138.52^{o} \pm 2.76$	↑ **28.79**
Unfermented-Control					
W	UD	$27.86^{klm} \pm 1.32$	–	$71.67^{abc} \pm 3.24$	–
	SD	$27.58^{klm} \pm 0.42$	↓ 1.01	$69.23^{ab} \pm 0.68$	↓ 3.46
	pH C-SD	$18.36^{def} \pm 0.97$	↓ **41.11**	$82.19^{fg} \pm 5.56$	↑ **13.67**
	SDD	$20.12^{fgh} \pm 1.14$	↓ **32.26**^{s}	$74.81^{bcd} \pm 4.01$	↑ 4.29^{ns}
	pH C-SDD	$11.94^{a} \pm 0.34$	↓ **80.00**	$76.21^{cde} \pm 1.79$	↑ 6.14

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

TFC of black tea increased from UD (75.05-103.66 mg CE/g) to 77.16-116.34 mg CE/g, 89.17-127.17 mg CE/g for SD and pH C-SD, respectively. The increase in TFC with SD was not considered biologically relevant, with the exception of sample P which had an increase greater than 10%. With SDD, TFC was 77.45-111.41 mg CE/g, with an increase compared to UD of 1.25-7.21. TFC for pH C-SDD was 67.14-138.52 mg CE/g. Green tea remained somewhat stable from UD (71.67 mg CE/g) to SD (69.23 mg CE/g) and SDD (74.81 mg CE/g) in terms of TFC. An increase in TFC was seen with pH C-SD (82.19 mg CE/g) and pH C-SDD (76.21 mg CE/g) treatments of green tea.

To summarize, TFC of black tea expressed as mg CE/ml remained relatively stable during digestion, with a 4.35% increase with SD, and a 1.10% increase with SDD compared to UD tea (Table 5.5). For adult digestion a decrease in TFC of 2.25% was found for both SD and SDD. As for TFC these differences between teas are not biologically relevant.

The increase in TPC and to a lesser extent TFC of black tea during SD could be as a result of increased extraction of polyphenolics or enzymatic degradation of larger polyphenolics such as flavonoids into smaller functional entities (Cilla *et al*., 2011, Khoddami *et al*., 2013). Acid and alkaline hydrolysis can also liberate phenolic compounds and flavonoids from their glycosides and aglycones (Cai *et al*., 2004). A synergistic increase in oxidative stability may also have been caused by the enzymes that were bound to phenolic compounds (Almajano, Delgado & Gordon, 2007), where both enzymes and phenolic compounds exhibit antioxidant properties. Green tea phenolic and flavonoid content was likely not affected by SD (TPC and TFC) or SDD (only TFC), which means that during these treatments, phenolic and flavonoid content remained relatively stable. Both black and green tea decreased in TPC at pH 4, SDD and pH 7. It is possible that black and green tea polyphenols and flavonoids are more sensitive to pH changes, particularly alkaline pH, than it is to enzymatic action. TPC values for SDD of black and green tea were slightly higher than that for pH 7, indicating that enzymes may have offered somewhat of a protective effect against neutral pH conditions that cause oxidation and degradation. TFC of black and green tea increased at pH 4 and pH 7 controls. Dimeric or oligomeric flavonoids may be broken down or hydrolysed into monomeric flavonoids, thereby increasing flavonoid content (Khoddami *et al*., 2013). TFC of black tea remained stable with SDD, possibly due to protective effects of enzymes on polyphenolic compounds as a result of binding. The results from this study are similar to that of Su *et al*. (2003), who observed that catechins and theaflavins are relatively stable at pH 4, however some degradation does occur, and are very unstable under neutral conditions.

Cilliers and Singleton (1989) found that oxidation of phenolic compounds occurs quite rapidly at higher pH. Record and Lane (2001) also found that black and green tea catechins were drastically reduced when incubated at pH 7 for 15 to 60 minutes, as a result of degradation under neutral conditions. Although TPC in the present study reflects these observations, TFC determination with the $AlCl_3$ assay provides very different results.

5.4.1.2 TPC and TFC – Aspalathus linearis

TPC and TFC of fermented and unfermented Rooibos tea digests were evaluated (Table 5.2). TPC of fermented Rooibos tea increased from 7.62-9.55 mg GAE/g for UD to 9.61-12.11 mg GAE/g for SD, in contrast to the decrease in TPC for pH C-SD (7.18-8.90 mg GAE/g). TPC decreased significantly with SDD (5.71-7.04 mg GAE/g) and pH C-SDD (6.22-7.16 mg GAE/g) treatments.

Unfermented Rooibos tea remained relatively stable from UD (12.09 mg GAE/g) to SD (12.52 mg GAE/g) in terms of TPC. A significant decrease in TPC was found for pH C-SD (8.46 mg GAE/g) and pH C-SDD (6.11 mg GAE/g) as well as SDD (8.33 mg GAE/g).

In summary, Table 5.5 shows a significant increase of 20% with SD, and a significant decrease of 25% with SDD. For adult digestion as indicated in Table 4.5, TPC was increased by 10.53% with SD but was unchanged for SDD. Therefore at pH 4, extraction of polyphenolics was increased, but subsequent reduction in pH resulted in a significant loss of TPC.

The TFC of UD, fermented Rooibos tea varied from 49.64-69.57 mg CE/g and remained stable with SD (50.56-63.48 mg CE/g) and pH C-SDD (42.84-73.85 mg CE/g). TFC of pH C-SD increased to 54.90-79.99 mg CE/g, whereas a slight decrease was observed with SDD (49.34-60.18 mg CE/g). TFC of unfermented Rooibos tea remained stable for UD (68.56 mg CE/g) and pH C-SD (68.75 mg CE/g) but was increased for pH C-SDD (74.16 mg CE/g). TFC decreased with SD and SDD to 55.96 mg CE/g and 60.99 mg CE/g, respectively.

In summary, TFC of fermented Rooibos tea remained relatively stable and only decreased by 1.71% and 5.22% with SD and SDD, respectively (Table 5.5). In contrast, with adult digestion (Table 4.5) TFC decreased by 20.56% and 10.71% for SD and SDD compared to UD respectively.

Table 5.2: Antioxidant content of undigested and infant digested unfermented and fermented *Aspalathus linearis* tea

Sample		Total Polyphenolic Content		Total Flavonoid Content	
		mg GAE/g	% Change	mg CE/g	% Change
Fermented					
F	UD	$7.62^{d} \pm 0.30$	–	$49.64^{b} \pm 2.17$	–
	SD	$9.61^{g} \pm 0.16$	↑ **23.10**	$50.56^{bc} \pm 1.12$	↑ 1.84
	pH C-SD	$7.18^{c} \pm 0.05$	↓ **5.95**	$54.90^{cd} \pm 3.68$	↑ **10.06**
	SDD	$5.71^{a} \pm 0.19$	↓ **28.66**^{s}	$49.34^{b} \pm 1.47$	↓ 0.61^{ns}
	pH C-SDD	$6.22^{b} \pm 0.14$	↓ **20.23**	$42.84^{a} \pm 1.09$	↓ **14.71**
L	UD	$9.25^{fg} \pm 0.34$	–	$69.57^{hi} \pm 2.71$	–
	SD	$12.11^{i} \pm 0.34$	↑ **26.78**	$63.48^{g} \pm 1.14$	↓ 9.15
	pH C-SD	$8.90^{f} \pm 0.37$	↓ 3.86	$79.99^{j} \pm 4.39$	↑ **13.93**
	SDD	$6.82^{c} \pm 0.12$	↓ **30.24**^{s}	$60.18^{efg} \pm 2.16$	↓ **14.47**^{ns}
	pH C-SDD	$7.16^{c} \pm 0.12$	↓ **25.47**	$73.85^{i} \pm 2.19$	↑ 5.97
P	UD	$9.55^{g} \pm 0.37$	–	$58.70^{defg} \pm 4.53$	–
	SD	$11.61^{h} \pm 0.10$	↑ **19.47**	$60.51^{efg} \pm 0.73$	↑ 3.04
	pH C-SD	$7.79^{d} \pm 0.10$	↓ **20.30**	$70.77^{hi} \pm 7.32$	↑ **18.65**
	SDD	$7.04^{c} \pm 0.27$	↓ **30.26**^{s}	$57.01^{def} \pm 1.69$	↓ 2.92^{ns}
	pH C-SDD	$6.37^{b} \pm 0.28$	↓ **39.95**	$60.26^{efg} \pm 3.96$	↑ 2.62
Unfermented-Control					
L	UD	$12.09^{i} \pm 0.50$	–	$68.56^{h} \pm 0.39$	–
	SD	$12.52^{j} \pm 0.14$	↑ 3.49	$55.96^{de} \pm 1.98$	↓ **20.24**
	pH C-SD	$8.46^{e} \pm 0.12$	↓ **35.33**	$68.75^{h} \pm 2.38$	↑ 0.28
	SDD	$8.33^{e} \pm 0.16$	↓ **36.83**^{s}	$60.99^{fg} \pm 1.82$	↓ **11.69**^{s}
	pH C-SDD	$6.11^{ab} \pm 0.22$	↓ **65.71**	$74.16^{i} \pm 1.98$	↑ 7.85

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)
Antioxidant activity multiply by 4 to convert to mmol/L

According to Standley *et al*. (2001), fermented Rooibos tea has a lower antioxidant capacity than unfermented Rooibos tea, due to the oxidation process of fermentation that results in lower amounts of flavonoids, particularly aspalathin. pH driven hydrolysis (Cai *et al*., 2004; Khoddami *et al*., 2013) of polyphenolics at pH 4 may account for the decrease in TPC for C pH-SD, however in the presence of pepsin an increase in TPC is found. Binding of polyphenls to protein may protect against acid dependent hydrolysis (Bandyopadhyay *et al*., 2012). Even though flavonoids are in general unstable under neutral and alkaline conditions, it is possible that alkaline hydrolysis could have liberated flavonoids from their glycosides

and aglycones (Cai *et al*., 2004), thereby maintaining antioxidant content even though some flavonoids may have been oxidised or degraded. TFC of fermented Rooibos tea did not change significantly with digestion which indicates that the flavonoids in *Aspalathus linearis* tea are not extracted to a significant degree or have undergone hydrolysis. Although only a single tea sample was evaluated, the increase in TFC following SDD of unfermented Rooibos tea may be due to increased hydrolysis of flavonoids from their glycosides and aglycones (Cai *et al*., 2004).

Viljoen (2008, pp. 177-178) reported that aspalathin, iso-orientin and orientin of fermented and unfermented Rooibos tea remained stable at pH 4, and decreased significantly at pH 7. This correlates with the TPC data of this study, where TPC remained stable or increased under acidic conditions but decreased in neutral pH conditions. In contrast, TFC remained unchanged.

5.4.2 Antioxidant activity of Camellia sinensis: DPPH, TEAC and ORAC

5.4.2.1 DPPH

Radical scavenging ability of UD black tea was 121.0-129.27 mg TE/g, and remained stable with SD (128.81-130.54 mg TE/g) (Table 5.3). A significant decrease in antioxidant activity of black tea was seen with SDD, which decreased to 77.74-110.27 mg TE/g. Radical scavenging ability decreased by 34.80-44.74% for pH C-SD, and 66.65-136.60% for pH C-SDD, which shows that a greater loss of radical scavenging ability of black tea occurred at neutral pH 7.

Radical scavenging ability of green tea displayed a similar trend to that of black tea, however was more affected by neutral pH conditions. Green tea remained stable from undigested (129.88 mg TE/g) to SD (132.72 mg TE/g), and decreased significantly to 88.88 mg TE/g with SDD. Radical scavenging ability of green tea decreased significantly with pH C-SD and pH C-SDD treatments by 42.62% and 131.69%, respectively. Black and green tea displayed similar values for radical scavenging ability, with the exception of pH C-SDD treatment, in which black tea had higher values.

To summarize, Table 5.5 shows a non-significant increase of 3.13% with SD of black tea, and a significant decrease of 32.26% following SDD compared to UD black tea. For adult digestion SD and SDD antioxidant activity decreased by 31.19% and 62.50% respectively.

Black and green tea radical scavenging ability remained stable with SD, however decreased significantly with SDD and pH 4 and pH 7 controls. The likelihood exists that digestive enzymes protected polyphenolic compounds during SD, as well as to some extent during SDD, through binding to the polyphenolic compounds and thereby nullifying or decreasing the effect that pH had on the structure of these compounds (Bandyopadhyay *et al*., 2012).

Table 5.3: Antioxidant activity of undigested and infant digested fermented and unfermented *Camellia sinensis* tea

Sample		DPPH		TEAC (mg TE/g)		ORAC (mg TE/g)	
		mg TE/g	% Change	mg TE/g	% Change	mg TE/g	% Change
Fermented							
W	UD	$121.08^{i} \pm 6.76$	–	$47.18^{bc} \pm 1.95$	–	$1162.10^{e} \pm 20.61$	–
	SD	$129.33^{jk} \pm 3.55$	↑ 6.59	$40.10^{a} \pm 4.51$	↓ **16.22**	$906.44^{d} \pm 69.21$	↓ **24.72**
	pH C-SD	$81.71^{e} \pm 3.54$	↓ **38.83**	$85.16^{ijk} \pm 4.13$	↑ **57.40**	$700.33^{bc} \pm 1.95$	↓ **49.59**
	SDD	$77.74^{e} \pm 3.29$	↓ **43.60**^{s}	$46.75^{b} \pm 2.45$	↓ 0.92^{s}	$628.29^{ab} \pm 77.14$	↓ **59.63**^{s}
	pH C-SDD	$22.79^{a} \pm 2.24$	↓ **136.60**	$87.35^{k} \pm 4.28$	↑ **59.72**	$699.89^{bc} \pm 5.14$	↓ **49.65**
G	UD	$123.16^{ij} \pm 5.99$	–	$50.03^{bc} \pm 0.38$	–	$1208.33^{e} \pm 33.85$	–
	SD	$130.54^{k} \pm 4.80$	↑ 5.82	$46.87^{b} \pm 2.78$	↓ 6.52	$959.51^{d} \pm 88.77$	↓ **22.96**
	pH C-SD	$82.09^{e} \pm 3.67$	↓ **40.02**	$88.55^{k} \pm 6.07$	↑ **55.59**	$692.36^{bc} \pm 5.36$	↓ **54.29**
	SDD	$80.69^{e} \pm 0.79$	↓ **41.67**^{s}	$49.18^{bc} \pm 1.45$	↓ 1.71^{ns}	$673.66^{abc} \pm 77.35$	↓ **56.82**^{s}
	pH C-SDD	$42.47^{b} \pm 6.05$	↓ **97.43**	$80.17^{ij} \pm 7.38$	↑ **46.30**	$709.86^{bc} \pm 10.14$	↓ **51.97**
F	UD	$129.27^{jk} \pm 5.34$	–	$61.48^{efg} \pm 2.42$	–	$1196.71^{e} \pm 8.40$	–
	SD	$128.81^{jk} \pm 4.42$	↓ 0.36	$53.15^{cd} \pm 2.80$	↓ **14.53**	$915.19^{d} \pm 65.70$	↓ **26.66**
	pH C-SD	$82.01^{e} \pm 3.99$	↓ **44.74**	$96.88^{mn} \pm 4.69$	↑ **44.71**	$714.81^{bc} \pm 8.59$	↓ **50.42**
	SDD	$95.41^{g} \pm 2.65$	↓ **30.14**^{s}	$60.00^{ef} \pm 1.62$	↓ 2.44^{s}	$717.14^{bc} \pm 97.66$	↓ **50.12**^{s}
	pH C-SDD	$52.44^{c} \pm 3.44$	↓ **84.56**	$89.96^{kl} \pm 2.90$	↑ **37.61**	$712.50^{bc} \pm 13.15$	↓ **50.72**
P	UD	$129.21^{jk} \pm 5.50$	–	$67.19^{g} \pm 1.83$	–	$1204.21^{e} \pm 2.58$	–
	SD	$130.12^{k} \pm 3.96$	↑ 0.70	$56.46^{de} \pm 3.22$	↓ **17.36**	$942.01^{d} \pm 86.22$	↓ **24.43**
	pH C-SD	$82.78^{ef} \pm 3.50$	↓ **34.80**	$102.25^{n} \pm 4.01$	↑ **41.38**	$726.98^{c} \pm 13.33$	↓ **49.42**
	SDD	$110.27^{h} \pm 4.13$	↓ **15.82**^{s}	$73.51^{h} \pm 3.96$	↑ 8.98^{s}	$734.49^{c} \pm 94.26$	↓ **48.46**^{s}
	pH C-SDD	$64.62^{d} \pm 3.89$	↓ **66.65**	$94.92^{lm} \pm 2.87$	↑ **34.21**	$721.07^{bc} \pm 24.69$	↓ **50.19**
Unfermented-Control							
W	UD	$129.88^{jk} \pm 4.28$	–	$51.25^{bcd} \pm 3.74$	–	$1123.11^{e} \pm 74.63$	–
	SD	$132.72^{k} \pm 3.77$	↑ 2.16	$46.50^{b} \pm 1.72$	↓ 9.72	$886.78^{d} \pm 83.55$	↓ **23.52**
	pH C-SD	$84.25^{ef} \pm 3.14$	↓ **42.62**	$74.78^{hi} \pm 5.11$	↑ **37.34**	$670.84^{abc} \pm 3.47$	↓ **50.42**
	SDD	$88.88^{fg} \pm 3.79$	↓ **37.48**^{s}	$37.27^{a} \pm 1.28$	↓ **31.59**^{s}	$584.69^{a} \pm 108.58$	↓ **63.05**^{s}
	pH C-SDD	$26.75^{a} \pm 0.76$	↓ **131.69**	$65.89^{fg} \pm 4.46$	↑ **25.00**	$730.08^{c} \pm 20.13$	↓ **42.42**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)
Antioxidant activity multiply by 4 to convert to mmol/L

Nanjo *et al*. (1996) found that the antioxidant activity of (+)-C and (-)-EC was reduced at pH 4 and was reported in several studies to be further reduced at pH 7 when measured with the

DPPH assay (Wootton-Beard *et al*., 2011; Chen *et al*., 2013). This may be due to the instability and consequent structural transformation or degradation of these polyphenols under these conditions (Chen *et al*., 2013; Zhu *et al*., 1997).

5.4.2.2 TEAC

The TEAC assay is widely used to measure the antioxidant activity of food products as this assay is stable to pH and can be used to study pH effects on activity (Zulueta *et al*., 2009). Antioxidant activity of UD black tea was 47.18-67.19 mg TE/g, as measured by the TEAC assay (Table 5.3). This decreased slightly during SD to 40.10-56.46 mg TE/g, and increased following SDD to 46.75-73.51 mg TE/g. Antioxidant activity increased by 41.38-57.40% following pH C-SD, and by 34.21-59.72% following pH C-SDD.

Digestion of green tea followed a similar trend as black tea, however the effect of SDD was greater than for black tea. For UD, antioxidant activity was 51.25 mg TE/g and with SD was 46.50 mg TE/g which decreased with SDD to 37.27 mg TE/g. Antioxidant activity of green tea increased significantly to 74.78 mg TE/g with pH C-SD, and then decreased slightly to 65.89 mg TE/g following pH C-SDD.

Compared to pH C-SD, both types of tea had lower antioxidant activity. Polyphenolics can bind protein and can alter antioxidant activity (Bandyopadhyay *et al*., 2012). Stojadinovic *et al*. (2013) investigated the interaction between dietary polyphenols such as those found in tea and β-lactoglobulin. Protein-polyphenol interaction protected protein secondary structure at pH 1.2 and 2.5 however this interaction masked total antioxidant capacity measured with the TEAC assay. The complex that forms will reduce the electron donating capacity and will reduce the number of available hydroxyl groups. Antioxidant activity of pH C-SDD was greater than SDD indicating even at a neutral pH, tea associated polyphenols bind protein. However polyphenol degradation at neutral pH results in decreased antioxidant activity.

In summary with infant digestion of *Camellia sinensis* tea, SD causes a 13.33% decrease and SDD a 1.77% increase in antioxidant activity measured with the TEAC assay. In contrast with adult digestion the decrease in antioxidant activity is 24.00% and 5.50% following SD and SDD respectively. For both adult and infant digestion, only SD causes a significant decrease in antioxidant activity.

5.4.2.3 ORAC

The ORAC assay measures the ability of antioxidants to protect fluorescein against the oxidative effects of the biologically relevant free radicals such as the peroxyl radical. The ORAC assay is pH sensitive (Zulueta *et al.* 2009), however the dilution of all solutions reduces the effect of pH. Furthermore as the ORAC assay is a fluorometric assay compared to the DPPH and TEAC assays that are colorimetric assays, extensive dilution of samples also reduces the effect of pH.

Antioxidant activity of black tea as measured by the ORAC assay decreased from 1162.10-1208.33 mg TE/g for UD to 906.44-959.51 mg TE/g for SD (Table 5.3) and then to 628.29-734.49 mg TE/g for SDD. The antioxidant activity for pH C-SD was 692.36-726.98 mg TE/g and for H C-SDD was 699.89-721.07 mg TE/g.

Green tea decreased in antioxidant activity from 1123.11 mg TE/g for UD to 886.78 mg TE/g for SD and 584.69 mg TE/g for SDD. For pH C-SD and pH C-SDD and oxidant activity was 670.84 mg TE/g and 730.08 mg TE/g respectively.

To summarize as indicated in Table 5.5 antioxidant activity measured with the ORAC assay decreased by 24.67% and 53.69% for SD and SDD respectively. For adult digestion this effect was 13.32% and 37.77% for SD and SDD respectively. In conclusion a greater loss in antioxidant activity was found following infant digestion.

In the ORAC assay AAPH is used to generate peroxyl radicals which cause the decomposition of fluorescein. Polyphenolics such as those found in tea protects fluorescein against the oxidative effects of peroxyl radicals by scavenging these radicals. With digestion polyphenolics can bind protein thereby masking antioxidant activity or alternatively peroxyl radicals can react with protein (Roche *et al.*, 2008) such as the digestive enzymes thereby reducing the effective concentration of peroxyl molecules available that can react with fluorescein. The latter effect may be the reason why antioxidant activity for SD and SDD is higher than pH C-SD and pH C-SDD.

5.4.3 Antioxidant activity of Aspalathus linearis: DPPH, TEAC and ORAC

5.4.3.1 DPPH

In fermented Rooibos tea the radical scavenging activity measured with the DPPH assay increased from 39.86-52.82 mg TE/g for UD tea to 48.17-56.19 mg TE/g for SD (Table 5.4), which then decreased following SDD to 33.61-40.87 mg TE/g. For the pH controls activity

was 29.53-37.81mg TE/g and 15.95-25.02 mg TE/g for pH C-SD and pH C-SDD respectively. Differences between pH C-SD and SD as well as pH C-SDD and SDD were significant for all samples.

For unfermented Rooibos tea, with SD antioxidant activity did not increase while for SDD activity decreased from 64.40 mg TE/g to 30.34 mg TE/g. Significant differences were also found between pH C-SD and SD as well as pH C-SDD and SDD with activity of SD and SDD being significantly greater than the pH controls.

In summary, antioxidant activity measured with the DPPH assay of digested fermented Rooibos tea increased significantly by 14.14% with SD and decreased by 21.69% following SDD, compared to the undigested value (Table 5.5).

Table 5.4: Antioxidant activity of undigested and infant digested unfermented and fermented *Aspalathus linearis* tea

Sample		DPPH		TEAC		ORAC	
		mg TE/g	% Change	mg TE/g	% Change	mg TE/g	% Change
Fermented							
F	UD	$39.86^{efg} \pm 1.08$	–	$16.63^{bcd} \pm 1.09$	–	$555.37^{g} \pm 24.95$	–
	SD	$48.17^{h} \pm 1.30$	↑ **18.88**	$13.53^{a} \pm 1.31$	↓ **20.56**	$388.87^{def} \pm 30.12$	↓ **35.27**
	pH C-SD	$29.53^{c} \pm 1.35$	↓ **29.77**	$24.43^{f} \pm 2.00$	↑ **37.99**	$323.46^{ab} \pm 4.52$	↓ **52.78**
	SDD	$33.61^{d} \pm 0.98$	↓ **17.01**^{s}	$15.82^{abc} \pm 0.67$	↓ 4.99^{ns}	$358.16^{bcde} \pm 39.38$	↓ **43.18**^{ns}
	pH C-SDD	$15.95^{a} \pm 0.93$	↓ **85.68**	$25.14^{fg} \pm 2.61$	↑ **40.75**	$308.63^{a} \pm 5.37$	↓ **57.12**
L	UD	$52.82^{i} \pm 2.43$	–	$20.88^{e} \pm 1.49$	–	$608.00^{h} \pm 34.12$	–
	SD	$56.19^{j} \pm 2.34$	↑ 6.18	$17.90^{bcde} \pm 0.75$	↓ **15.37**	$428.46^{f} \pm 39.42$	↓ **34.64**
	pH C-SD	$37.81^{e} \pm 1.63$	↓ **33.12**	$32.44^{jk} \pm 3.73$	↑ **43.46**	$341.29^{abc} \pm 3.93$	↓ **56.19**
	SDD	$40.87^{fg} \pm 3.61$	↓ **25.51**^{s}	$19.63^{de} \pm 1.95$	↓ 6.17^{ns}	$370.24^{bcde} \pm 50.10$	↓ **48.61**^{s}
	pH C-SDD	$25.02^{b} \pm 1.31$	↓ **71.43**	$29.26^{hi} \pm 2.64$	↑ **33.43**	$332.10^{ab} \pm 17.06$	↓ **58.70**
P	UD	$45.92^{h} \pm 1.39$	–	$19.63^{de} \pm 1.19$	–	$573.70^{gh} \pm 21.46$	–
	SD	$54.89^{ij} \pm 2.14$	↑ **17.80**	$16.87^{bcd} \pm 0.91$	↓ **15.12**	$393.64^{ef} \pm 34.33$	↓ **37.23**
	pH C-SD	$37.16^{e} \pm 1.60$	↓ **21.09**	$33.82^{k} \pm 2.26$	↑ **53.10**	$334.15^{ab} \pm 3.54$	↓ **52.77**
	SDD	$38.01^{ef} \pm 1.34$	↓ **18.85**^{s}	$17.71^{bcd} \pm 1.00$	↓ **10.28**^{ns}	$367.26^{bcde} \pm 38.42$	↓ **43.88**^{ns}
	pH C-SDD	$18.34^{a} \pm 0.25$	↓ **85.84**	$27.55^{gh} \pm 2.83$	↑ **33.57**	$338.54^{abc} \pm 10.32$	↓ **51.56**
Unfermented-Control							
L	UD	$64.30^{k} \pm 1.92$	–	$29.87^{hij} \pm 1.27$	–	$600.98^{gh} \pm 35.46$	–
	SD	$64.40^{k} \pm 1.88$	↑ 0.16	$18.87^{cde} \pm 1.25$	↓ **45.14**	$385.55^{cdef} \pm 39.47$	↓ **43.67**
	pH C-SD	$41.39^{g} \pm 1.74$	↓ **43.35**	$31.31^{ijk} \pm 1.97$	↑ 4.71	$344.19^{abcd} \pm 4.53$	↓ **54.34**
	SDD	$30.34^{c} \pm 1.85$	↓ **71.77**^{s}	$15.57^{ab} \pm 0.34$	↓ **62.94**^{s}	$347.55^{abcde} \pm 39.29$	↓ **53.44**^{ns}
	pH C-SDD	$17.64^{a} \pm 0.82$	↓ **113.89**	$24.36^{f} \pm 2.06$	↓ **20.32**	$366.99^{bcde} \pm 11.05$	↓ **48.35**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*
Antioxidant activity multiply by 4 to convert to mmol/L

Unfermented Rooibos tea remained stable during SD, as opposed to the decrease observed at pH C-SD. The same trend was seen with SDD digests of fermented and unfermented Rooibos tea having higher values than that at pH 7. It is therefore possible that enzymes protected polyphenolic compounds from pH conditions by binding to them (Cilla *et al*., 2011), resulting in radical scavenging activity that was higher than the digests' corresponding pH control. Radical scavenging by digestive enzyme proteins (Roche *et al*., 2008) could also have been the reason for SD and SDD having higher radical scavenging ability than their respective pH controls.

The antioxidant activity of fermented Rooibos tea increased with SD compared to UD which implies increased polyphenolic extraction. The presence of the proteolytic enzymes prevents pH driven degradation as seen with the pH C-SD. Even though the oxidation process is slow under acidic conditions, it does occur (Cilliers & Singleton, 1989). This could explain the decrease in radical scavenging ability of fermented and unfermented Rooibos tea observed at pH 4.

Neutral pH conditions promote oxidation and degradation of polyphenolic compounds (Ferruzzi, 2010; Spencer, 2003; Green *et al*., 2007; Viljoen, 2008), resulting in a lowering of radical scavenging ability. This is seen for fermented and unfermented Rooibos tea where compared to UD the average decrease in antioxidant activity for fermented Rooibos was 71.43-85.84% and 17.01-25.51% for pH C-SDD and SDD respectively, and for unfermented Rooibos 113.89% and 71.77% for pH C-SDD and SDD respectively. Again the presence of proteolytic enzymes was shown to effectively reduce pH associated degradation. Remaining activity following digestion was 80.43% for fermented Rooibos tea.

5.4.3.2 TEAC

The antioxidant activity, as measured by the TEAC assay, of UD fermented Rooibos tea was 16.63-20.88 mg TE/g, which decreased slightly in antioxidant activity following SD (13.53-17.90 mg TE/g) and SDD (15.82-19.63 mg TE/g) (Table 5.4). Differences between UD and SD was significant for F but not for L and P. At pH 4 for pH C-SD antioxidant activity was increased for pH C-SD by 37.99-53.10% compared to UD. In contrast to the DPPH assay generated data, SD levels were less than pH C-SD. Likewise with further digestion pH C-SDD levels (25.14-29.26 mg TE/g) were significantly greater than SDD (15.82-19.63 mg TE/g) levels.

Antioxidant activity of UD unfermented Rooibos tea (29.87 mg TE/g) decreased significantly during SD and SDD to 18.87 mg TE/g and 15.57 mg TE/g respectively. Similar to fermented Rooibos tea, levels of antioxidant activity were greater for pH C-SD and pH C-SDD when compared to SD and SDD.

In summary antioxidant activity for fermented Rooibos tea compared to UD decreased by 17.14% for SD and 5.41% for SDD (not significant). In contrast with the DPPH assay antioxidant activity was increased for pH C-SD and pH C-SDD. Remaining activity following digestion was 94.74%.

Rooibos tea is a complex mixture of unique polyphenolics which may have variable sensitivity to pH dependent degradation or increased extraction of these molecules may occur at low pH (Khoddami *et al*., 2013; Cai *et al*., 2004), while the protein that is present may protect against degradation. The observed effects are the sum of all these processes.

5.4.3.3 ORAC

In the ORAC assay it was observed that UD fermented Rooibos tea (555.37-608.00 mg TE/g) antioxidant activity decreased for SD (388.87-428.46 mg TE/g) and SDD (358.16-370.24 mg TE/g). Differences between SD and SDD were significant for L but not F and P. The antioxidant activity measured for pH C-SD (323.46-341.29 mg TE/g), and pH C-SDD (308.63-338.54 mg TE/g) (Table 5.4) was for all samples significantly less than SD but for SDD only for sample F. Remaining antioxidant activity following complete digestion was 63.04%.

The antioxidant activity of UD unfermented Rooibos tea was 600.98 mg TE/g which with SD and SDD decreased to 385.55 mg TE/g and 347.55 mg TE/g respectively. Differences between SD and SDD were not significant.

In summary, SD and SDD caused a 35.61% and 45.34% decrease in antioxidant activity measured with the ORAC assay (Table 5.5). Differences between SD and SDD are not significant.

Similar to *Camellia sinensis* tea at pH 4, digestion caused a decrease in antioxidant activity measured with the ORAC assay. Therefore the polyphenolic compounds found in Rooibos tea are sensitive and unstable at acidic and/or neutral pH conditions. Inclusion of the digestive enzymes reduces degradation as was also found with the DPPH antioxidant assay. In the DPPH assay it may be due to the gastric enzymes as proteins protecting against acid

driven polyphenolic degradation or in the ORAC the scavenging of radicals by proteins (Roche *et al*., 2008). Viljoen (2008, pp. 177-178) reported that at pH 4, aspalathin, orientin and iso-orientin that are found in Rooibos tea are stable. However in this study antioxidant activity measured with the TEAC and ORAC assay was reduced while activity measured with the DPPH assay was increased.

Viljoen (2008, pp. 177-178) also observed that at pH 6 and 7, aspalathin, orientin and iso-orientin content of fermented and unfermented Rooibos tea decreased. With SDD antioxidant activity of fermented Rooibos tea was reduced by 21.69%, 5.41% and 45.34% for the DPPH, TEAC and ORAC assays respectively (Table 5.5).

Differences in antioxidant activity measured for UD, SD and SDD is a function of the principles of the assay, the effect of other included molecules e.g. salts and protein, pH as well as the type, stability and activity of polyphenolics found in each sample.

Remaining activity between adult (Table 4.5) and infant (Table 5.5) digestion was 67.39% vs 80.43% (significant, DPPH assay), 89.49% vs 94.74% vs (not significant, TEAC assay) and 65.63% vs 63.04% (significant, ORAC assay). Based on the definition of biological relevance, pH differences in SD between adult and infant digestion does not result in significant differences in antioxidant activity.

Table 5.5: Summary of antioxidant properties of undigested and infant digested fermented and unfermented *Camellia sinensis* and *Aspalathus linearis* tea per ml

Sample	TPC		TFC		DPPH		TEAC		ORAC	
	mg GAE/ml	% Change	mg CE/ml	% Change	mg TE/ml	% Change	mg TE/ml	% Change	mg TE/ml	% Change
Black Tea										
UD	$0.28^{e} \pm 0.02$	–	$0.90^{b} \pm 0.12$	–	$1.26^{b} \pm 0.04$	–	$0.56^{b} \pm 0.08$	–	$11.93^{e} \pm 0.24$	–
SD	$0.33^{f} \pm 0.05$	↑ **16.39**	$0.94^{b} \pm 0.17$	↑ 4.35	$1.30^{b} \pm 0.01$	↑ 3.13	$0.49^{c} \pm 0.07$	↓ **13.33**	$9.31^{d} \pm 0.24$	↓ **24.67**
SDD	$0.20^{d} \pm 0.03$	↓ **33.33**^{s}	$0.91^{b} \pm 0.15$	↑ 1.10^{ns}	$0.91^{d} \pm 0.15$	↓ **32.26**^{s}	$0.57^{b} \pm 0.12$	↑ 1.77^{s}	$6.88^{c} \pm 0.48$	↓ **53.69**^{s}
% Remaining content/activity	**71.43%**		**101.11%**		**72.22%**		**101.79%**		**57.67%**	
Fermented Rooibos Tea										
UD	$0.09^{b} \pm 0.01$	–	$0.59^{a} \pm 0.10$	–	$0.46^{a} \pm 0.06$	–	$0.19^{a} \pm 0.02$	–	$5.79^{b} \pm 0.27$	–
SD	$0.11^{c} \pm 0.01$	↑ **20.00**	$0.58^{a} \pm 0.07$	↓ 1.71	$0.53^{a} \pm 0.04$	↑ **14.14**	$0.16^{a} \pm 0.02$	↓ **17.14**	$4.04^{a} \pm 0.22$	↓ **35.61**
SDD	$0.07^{a} \pm 0.01$	↓ **25.00**^{s}	$0.56^{a} \pm 0.06$	↓ 5.22^{ns}	$0.37^{c} \pm 0.04$	↓ **21.69**^{s}	$0.18^{a} \pm 0.02$	↓ 5.41^{ns}	$3.65^{a} \pm 0.06$	↓ **45.34**^{ns}
% Remaining content/activity	**77.78%**		**94.92%**		**80.43%**		**94.74%**		**63.04%**	

Data is an average of each tea type ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)

***Bold** indicates biological relevant difference from UD (≥ 10% in % Change)*

$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

Antioxidant activity multiply by 4 to convert to mmol/L

5.4.4 Cellular and biological assays

5.4.4.1 NO scavenging ability of Camellia sinensis

UD black tea had the highest NO scavenging ability of 70.38-73.62%. Scavenging ability of black tea decreased with SD and SDD to 60.67-66.08% and 62.07-66.60%, respectively (Table 5.6). For pH C-SD and pH C-SDD the scavenging activity was 60.51 -68.06% and 31.46-60.44% respectively. The NO scavenging activity was greater for SD compared to pH C-SD and SDD compared to pH C-SDD which indicates as for antioxidant activity the presence of protein prevents pH driven degradation of polyphenolics or that the protein also has inherent NO scavenging ability. Biologically relevant decrease in NO scavenging ability was found for SDD of W, G and F teas. Activity that remained following SDD was high and was 60.44-68.06%.

Green tea had a similar trend to black tea. Undigested green tea had the highest NO scavenging ability of 66.39%, which decreased following SD (56.79%) and then remained stable following SDD (56.79%). A significant decrease of 41.25% in scavenging ability of green tea was found with pH C-SD. Scavenging ability of green tea decreased profoundly by 98.55% with pH C-SDD. Black tea had higher NO scavenging ability than green tea. Similar to black tea, significant differences in activity were found between green tea pH C-SD and SD as well as pH C-SDD and SDD.

In summary, NO scavenging activity decreased with digestion compared to UD by 12.89% and 11.33% for SD and SDD respectively (Table 5.10). Differences between SD and SDD were not significant.

NO scavenging ability of black and green tea did not have a similar trend to that of their TPC or TFC content. It is possible that during SD and SDD of black and green tea, polyphenolic compounds may have been protected by enzymes or broken down and released by enzymes (Cilla *et al*., 2011), which is why digests had higher scavenging ability than their respective controls. NO scavenging ability was significantly affected by pH conditions, perhaps as a result of polyphenolic compounds present in black and green teas that were less stable at acidic and/or neutral pH, which lead to oxidation and degradation of these compounds (Record & Lane, 2001). Hence, NO scavenging ability was reduced.

Table 5.6: NO scavenging ability of undigested and infant digested fermented and unfermented *Camellia sinensis* tea

Sample		NO Scavenged		
		% Scavenged	**TE (µM)**	**% Change**
Fermented				
W	UD	$70.38^{hij} \pm 2.53$	773.86	–
	SD	$60.67^{ef} \pm 1.79$	667.14	↓ **14.81**
	pH C-SD	$54.34^{d} \pm 3.43$	597.50	↓ **25.72**
	SDD	$62.07^{fg} \pm 3.41$	682.53	↓ **12.54**^{ns}
	pH C-SDD	$26.59^{b} \pm 3.06$	292.41	↓ **90.31**
G	UD	$71.19^{ij} \pm 1.30$	782.87	–
	SD	$62.74^{fg} \pm 1.49$	689.91	↓ **12.62**
	pH C-SD	$52.66^{d} \pm 3.98$	579.07	↓ **29.93**
	SDD	$64.37^{fg} \pm 2.72$	707.83	↓ **10.07**^{ns}
	pH C-SDD	$22.38^{b} \pm 2.83$	246.07	↓ **104.34**
F	UD	$73.12^{j} \pm 2.28$	804.03	–
	SD	$63.97^{fg} \pm 1.23$	703.37	↓ **13.36**
	pH C-SD	$53.52^{d} \pm 3.08$	588.56	↓ **30.95**
	SDD	$64.33^{fg} \pm 2.63$	707.33	↓ **12.80**^{ns}
	pH C-SDD	$8.05^{a} \pm 3.84$	88.52	↓ **160.33**
P	UD	$73.62^{j} \pm 1.36$	809.51	–
	SD	$66.08^{gh} \pm 0.51$	726.58	↓ **10.80**
	pH C-SD	$54.10^{d} \pm 3.76$	594.94	↓ **30.56**
	SDD	$66.60^{ghi} \pm 2.54$	732.40	↓ **10.00**^{ns}
	pH C-SDD	$54.24^{d} \pm 4.09$	596.39	↓ **30.32**
Unfermented-Control				
W	UD	$66.39^{ghi} \pm 4.43$	730.03	–
	SD	$56.79^{de} \pm 2.08$	624.47	↓ **15.59**
	pH C-SD	$43.69^{c} \pm 4.17$	480.38	↓ **41.25**
	SDD	$56.79^{de} \pm 5.07$	624.52	↓ **15.58**^{ns}
	pH C-SDD	$22.56^{b} \pm 2.39$	248.08	↓ **98.55**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)

***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*

$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

Neutral pH severely affected NO scavenging abilities of black and green teas. Various studies have found that tea antioxidants are more severely affected by near neutral pH conditions (Ferruzzi, 2010; Spencer, 2003; Green *et al*., 2007), and that oxidation of phenolic compounds occurs more rapidly at neutral/alkaline conditions (Cilliers & Singleton, 1989). This increase in oxidation of polyphenolic compounds present in black and green tea at neutral pH most likely caused NO scavenging ability to drastically decrease as well.

5.4.4.2 NO scavenging ability of Aspalathus linearis

NO scavenging ability of digested Rooibos tea was also determined (Table 4.7). UD scavenged 34.46-37.85% NO while SD and SDD scavenged 27.44-29.34% and 26.20-34.91% NO respectively. A more significant decrease in scavenging ability of fermented Rooibos tea was found with pH C-SD and pH C-SDD treatments, which decreased by 44.12-48.61% and 36.33-70.82%, respectively, from undigested fermented Rooibos tea values. The percentage decrease for all samples, except SDD of sample P, was biologically relevant.

Undigested unfermented Rooibos tea had the highest NO scavenging ability of 44.58%, which decreased following SD and SDD to 35.09% and 40.42%, respectively. A significant decrease of 40.94% in scavenging ability of unfermented Rooibos tea was seen with pH C-SD. Greatest loss in scavenging ability of unfermented Rooibos tea was observed with pH C-SDD (16.30%).

Table 5.10 summarizes that 25.70% of NO scavenging ability was lost with SD, which then increased by a non-significant 9.47% following SDD.

NO scavenging ability of fermented and unfermented Rooibos tea followed a similar trend to that of black and green tea in the assay, and also did not have a similar trend to that of their TPC or TFC contents. Polyphenolics have been described to scavenge NO (Duarte, Francisco & Perez-Vizcaino, 2014), therefore as described for antioxidant activity, an increase in pH can cause increased extraction while at a neutral pH polyphenol oxidation or degradation occurs (Cilliers & Singleton, 1989, Record & Lane, 2001). Furthermore the presence of protein such as proteolytic enzymes can digest the matrix to release polyphenolics (Cilla *et al*., 2011) or can trap or scavenge NO (Roche *et al*., 2008). Stojadinovic *et al*. (2013) observed that polyphenol-protein complexes form at acidic and neutral pH conditions of the GIT. These authors also noted a reduced radical scavenging activity of these complexes compared to their individual components.

Table 5.7: NO scavenging ability of undigested and infant digested fermented and unfermented *Aspalathus linearis* tea

Sample		NO Scavenged (%)		
		% Scavenged	TE (µM)	% Change
Fermented				
F	UD	$34.46^{hi} \pm 3.91$	378.92	–
	SD	$28.30^{efg} \pm 2.79$	311.16	↓ **19.64**
	pH C-SD	$22.00^{bc} \pm 2.32$	241.95	↓ **44.12**
	SDD	$26.20^{cdef} \pm 2.56$	288.05	↓ **27.25**^{ns}
	pH C-SDD	$23.86^{cde} \pm 2.44$	262.41	↓ **36.33**
L	UD	$37.80^{ij} \pm 4.18$	415.67	–
	SD	$29.34^{fg} \pm 3.18$	322.62	↓ **25.21**
	pH C-SD	$23.14^{cd} \pm 2.66$	254.44	↓ **48.12**
	SDD	$32.44^{gh} \pm 4.59$	356.74	↓ **15.26**^{ns}
	pH C-SDD	$23.08^{cd} \pm 2.46$	253.74	↓ **48.38**
P	UD	$37.85^{ij} \pm 4.18$	416.21	–
	SD	$27.44^{def} \pm 3.20$	301.73	↓ **31.89**
	pH C-SD	$23.05^{cd} \pm 1.42$	253.44	↓ **48.61**
	SDD	$34.91^{hi} \pm 0.63$	383.91	↓ 8.07^{s}
	pH C-SDD	$18.05^{ab} \pm 2.46$	198.52	↓ **70.82**
Unfermented-Control				
L	UD	$44.58^{k} \pm 5.21$	490.18	–
	SD	$35.09^{hi} \pm 2.83$	385.86	↓ **23.82**
	pH C-SD	$29.43^{fg} \pm 1.67$	323.59	↓ **40.94**
	SDD	$40.42^{jk} \pm 0.71$	444.49	↓ 9.78^{s}
	pH C-SDD	$16.30^{a} \pm 1.80$	179.20	↓ **92.92**

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

5.4.4.3 DCFH-DA of Camellia sinensis

In the Caco-2 cell line the DCFH-DA assay was then used to determine the ability of UD and infant digested teas to quench radicals and reduce or prevent oxidative damage. Tea has been reported to have a pro-oxidant effect (Lambert & Elias, 2010) in cell culture. To determine if the dilutions used caused a pro-oxidant effect the effect of each extract in the absence of AAPH was determined. For all samples no increase in fluorescence was observed which implies that the tea

extracts did not cause oxidative damage i.e. did not have a pro-oxidant effect (data not shown). The ability of each extract to prevent oxidative cellular damage was determined.

AAPH causes 100% damage while in the presence of UD black tea this decreased to 2.47-3.75%. Digestion resulted in 2.41-4.85% and 1.52-12.60% damage being measured for SD and SDD respectively. For pH C-SD and pH C-SDD differences were 3.73-4.78% and 5.15-7.14% for pH C-SD and pH C-SDD respectively. Differences between SD and pH C-SD were significant for samples W, G and F but not for P while differences between SDD and pH C-SDD was significant for all samples. SD and SDD showed a higher degree of cellular protection than pH C-SD and pH C-SDD in most samples. The percentage damage of green tea was negative for UD and pH C-SD indicating heightened protection. In future it may be necessary to dilute this sample further. SD and SDD reduced cellular damage to 2.83% and 2.36% respectively.

In summary, black tea and infant digests effectively protected the Caco-2 cells against oxidative damage. When reported as % protection/ml digestion caused no change in the cellular antioxidant activity of SD. Measured activity of SDD differed significantly from UD, although this difference was not biologically relevant.

No published literature could be found related to the effect of digestion on the cellular antioxidant activity of *Camellia sinensis* tea. Oxidation of phenolic compounds is normally slow under acidic conditions, however at higher pH occurs quite rapidly (Cilliers & Singleton, 1989). Record and Lane (2001) found that EGC, EGCG, GCG, and ECG of black tea were slightly lowered when incubated at acidic pH conditions while a drastic decrease in these catechins occurred at neutral pH. TPC, antioxidant activity (DPPH and ORAC assays) and NO scavenging ability decreased with SDD. The degree of cellular protection against AAPH induced oxidative damage was significantly different to the UD samples. Binding of polyphenols to protein (Bravo, 1998) would decrease the measured levels of cellular protection, whereas if the protein scavenged free radicals together with the scavenging ability of polyphenolics this would result in increased cellular antioxidant protection, as was found for SD vs pH C-SD and SDD vs pH C-SDD.

Table 5.8: Cellular protective effect of undigested and infant digested fermented and unfermented *Camellia sinensis* tea

Sample		DCFH-DA	
		% Damage	% Change
Fermented			
W	UD	$3.55^{gh} \pm 0.14$	–
	SD	$2.41^{g} \pm 0.23$	↓ **38.26**
	pH C-SD	$4.78^{i} \pm 0.38$	↑ **29.53**
	SDD	$3.74^{gh} \pm 0.02$	↑ 5.21^{ns}
	pH C-SDD	$5.97^{l} \pm 0.61$	↑ **50.84**
G	UD	$3.33^{fgh} \pm 0.30$	–
	SD	$3.09^{efg} \pm 0.13$	↓ 7.48
	pH C-SD	$3.80^{h} \pm 0.21$	↑ **13.18**
	SDD	$12.60^{n} \pm 1.04$	↑ **116.38**^{s}
	pH C-SDD	$5.14^{ijk} \pm 0.51$	↑ **42.74**
F	UD	$2.47^{de} \pm 0.22$	–
	SD	$4.85^{ij} \pm 0.50$	↑ **65.03**
	pH C-SD	$3.73^{gh} \pm 0.37$	↑ **40.65**
	SDD	$1.52^{c} \pm 0.10$	↓ **47.62**^{s}
	pH C-SDD	$5.69^{kl} \pm 0.17$	↑ **78.92**
P	UD	$3.75^{h} \pm 0.18$	–
	SD	$3.75^{h} \pm 0.31$	0
	pH C-SD	$3.82^{h} \pm 0.41$	↑ 1.85
	SDD	$2.27^{d} \pm 0.23$	↓ **49.17**^{s}
	pH C-SDD	$7.14^{m} \pm 0.56$	↑ **62.26**
Unfermented-Control			
W	UD	$-5.57^{a} \pm 0.62$	–
	SD	$2.83^{def} \pm 0.26$	*
	pH C-SD	$-4.65^{b} \pm 0.45$	*
	SDD	$2.36^{d} \pm 0.25$	*
	pH C-SDD	$5.47^{jkl} \pm 0.31$	*

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)

** Cannot be determined due to undigested and stomach control pH4 values being negative, hence having a pro-oxidant effect*

***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*

$^{s/ns}$ Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)

5.4.4.4 DCFH-DA of Aspalathus linearis

Compared to AAPH that causes 100% damage, UD Rooibos tea caused the oxidative effect of AAPH to be reduced to 14.67-16.64%. Digestion resulted in 13.39-23.03% and 13.46-18.31% damage being measured for SD and SDD respectively. Differences between UD and SD were significant for sample F but not for samples L and P. In contrast differences between UD and SDD were not significant for L, F and P. For pH C-SD and pH C-SDD differences were 17.88-22.47% and 20.23-31.22% for pH C-SD and pH C-SDD respectively. Differences between SD and pH C-SD were significant for samples L and P but not for F while differences between SDD and pH C-SDD was significant for all samples. As for black tea, SD and SDD of Rooibos tea showed a higher degree of cellular protection than pH C-SD and pH C-SDD. The percentage damage of green Rooibos tea was 13.16%, 15.60% and 14.50% for UD, SD and SDD respectively which was similar to the cellular antioxidative effects of fermented Rooibos tea.

To summarise with digestion Rooibos tea retains high levels of cellular antioxidant activity which does not decrease significantly with a 2.45% decrease and subsequent 1.67% increase in cellular antioxidant activity for SD and SDD respectively.

Although Viljoen (2008, pp. 177-178) reported that aspalathin, iso-orientin and orientin content of fermented Rooibos tea decreased significantly at pH 7, measured cellular antioxidant activity does not reflect this change in content. The presence of gastric enzymes also decreases oxidative damage caused by AAPH.

Black tea following digestion retained 94.97% cellular antioxidant activity following digestion while for Rooibos tea 83.99% cellular antioxidant activity was retained. While the effect of black tea was significantly greater than Rooibos tea, biologically activity is similar with a difference of 10.98%.

The % remaining activity of black tea was 83.15% and 94.97% and for Rooibos tea was 71.71% and 83.99% for adult and infant digestion respectively. For infants the % remaining activity was more than 10% greater than for adult digestion meaning differences are biologically relevant.

Table 5.9: Cellular protective effect of undigested and infant digested fermented and unfermented *Aspalathus linearis* tea

Sample		DCFH-DA	
		% Damage	% Change
Fermented			
F	UD	$16.64^{cde} \pm 1.54$	–
	SD	$23.03^{i} \pm 2.44$	↑ **32.22**
	pH C-SD	$22.47^{hi} \pm 0.64$	↑ **29.81**
	SDD	$18.31^{efg} \pm 1.30$	↑ 9.56^{s}
	pH C-SDD	$31.22^{j} \pm 2.97$	↑ **60.93**
L	UD	$14.74^{abc} \pm 1.12$	–
	SD	$15.76^{cd} \pm 1.10$	↑ 6.69
	pH C-SD	$19.15^{fg} \pm 1.31$	↑ **26.03**
	SDD	$16.26^{cde} \pm 1.29$	↑ 9.81^{ns}
	pH C-SDD	$22.57^{i} \pm 1.84$	↑ **41.97**
P	UD	$14.67^{abc} \pm 1.07$	–
	SD	$13.39^{ab} \pm 0.44$	↓ 9.12
	pH C-SD	$17.88^{def} \pm 0.77$	↑ **19.72**
	SDD	$13.46^{ab} \pm 1.20$	↓ 8.60^{ns}
	pH C-SDD	$20.23^{gh} \pm 0.91$	↑ **31.86**
Unfermented-Control			
L	UD	$13.16^{a} \pm 0.86$	–
	SD	$15.60^{bcd} \pm 0.24$	↑ **16.97**
	pH C-SD	$14.76^{abc} \pm 1.11$	↑ **11.46**
	SDD	$14.50^{abc} \pm 1.45$	↑ 9.69^{ns}
	pH C-SDD	$13.32^{ab} \pm 1.34$	↑ 1.21

Data is an average of 3 experiments ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change).*
$^{s/ns}$ Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)

Table 5.10: Summary of NO scavenging ability and the cellular protective effects of fermented *Camellia sinensis* and *Aspalathus linearis* tea per ml following infant digestion

Sample	NO Scavenging		DCFH-DA	
	mg TE/ml	% Change	% Protection	% Change
Black Tea				
UD	7. 93^{d} ± 0.08	–	96.72^{b} ± 0.05	–
SD	6. 97^{b} ± 0.11	↓ **12.89**	96.47^{b} ± 0.10	↓ 0.26
SDD	7.08^{b} ± 0.31	↓ **11.33**^{ns}	94.97^{d} ± 0.27	↓ 1.83^{s}
% Remaining activity	**89.28%**		**94.97%**	
Fermented Rooibos Tea				
UD	4.04^{c} ± 0.21	–	84.65^{a} ± 0.81	–
SD	3.12^{a} ± 0.10	↓ **25.70**	82.60^{c} ± 0.94	↓ 2.45
SDD	3.43^{a} ± 0.49	↓ **16.33**^{ns}	83.99^{a} ± 0.31	↓ 0.78^{s}
% Remaining activity	**84.90%**		**83.99%**	

Data is an average of each tea type ± SD. Mean values in column with different letters are significantly different ($p \leq 0.05$)
***Bold** indicates biological relevant difference from UD (≥ 10% in % Change)*
$^{s/ns}$ *Statistically significant/not significant difference between SD and SDD samples ($p \leq 0.05$)*

5.5 Conclusion

For black tea, infant SD with a low pH causes an increase in the extraction of polyphenolics which with further SDD decreases by 33.33% compared to UD. No changes in TFC levels were found with digestion. Antioxidant activity in the DPPH assay of digested black tea was unaltered for SD and for SDD reduced by 32.26% compared to UD. Remaining activity following digestion was 72.22%. With the TEAC assay the measured antioxidant activity decreased by 13.33% for SD and for SDD was unchanged compared to UD. Following SDD the remaining antioxidant activity was 101.79% of UD. With the ORAC assay antioxidant activity was reduced by 24.67% and 53.69% for SD and SDD respectively compared to UD. Remaining activity following SDD was 57.67%. NO scavenging activity of black tea was reduced by 12.89% and 11.33% for SD and SDD respectively compared to UD. Evaluation of cellular antioxidant activity revealed that activity was unchanged for SD and decreased slightly for SDD, with the remaining activity 94.97%.

For fermented Rooibos tea, SD resulted in an increase in TPC levels but with further digestion this resulted in a 25.00% decrease in TPC compared to UD. Remaining TPC was 77.78%. With digestion TFC levels were reduced to 94.92%. Antioxidant activity of digested Rooibos tea measured with the DPPH assay increased by 14.14% for SD and decreased by 21.69% for SDD

compared to UD. Remaining activity following digestion was 80.43%. With the TEAC assay with SD and SDD antioxidant activity decreased by 17.14% and 5.41% respectively compared to UD. Remaining activity was 94.74%. The ORAC assay revealed that antioxidant activity was reduced by 35.61% and 45.34% for SD and SDD respectively while the remaining activity compared to UD was 63.04%. Fermented Rooibos NO scavenging activity was 25.70% and 16.33% lower for SD and SDD respectively, compared to UD. Cellular antioxidant activity was decreased by 2.45% for SD and was unchanged for SDD with 83.99% protection activity remaining. Similar to adult digestion, the presence of proteins such as the digestive enzymes seem to modulate measured antioxidant activity either by possibly quenching free radicals or by directly binding polyphenols.

Black tea and Rooibos tea are both rich in polyphenolics although the composition and structure differ greatly. Black tea consistently showed a better activity than Rooibos tea; however both retain antioxidant activity, NO scavenging ability which results in cellular protection against oxidants.

Although digestion does alter antioxidant content and activity, NO scavenging ability and cellular antioxidant activity of both *Camellia sinensis* and *Aspalathus linearis* teas retained antioxidant properties demonstrating that these teas are affordable sources of antioxidants for infants when consumed as part of a healthy, balanced diet.

Chapter 6: General Discussion, Recommendations and Conclusions

6.1 Introduction

Vitamin A (retinol) is needed for optimal vision, growth and development, maintenance of epithelial tissue, reproduction and immune function. The estimated mean requirement of vitamin A for infants is 190 µg RE/day, for female adults is 270 µg RE/day, and for male adults is 300 µg RE/day. The role of Vitamin E in the body is to protect polyunsaturated fatty acids (PUFAs), other cell membrane components, and LDL from oxidative damage. The safe allowance for vitamin E is 10 mg/day for men and 7-8 mg/day for women. Vitamin C plays a role in enzymatic functions, collagen hydroxylation and antioxidant activity. The mean requirement for vitamin C is 25-30 mg/day (FAO/WHO, 2004). Vitamins A, C and E are compounds that possess antioxidant potential, which defend against free-radical damage (Whitney & Rolfes, 2008, p. 391). Sources include whole milk, glandular meats, liver, fish liver oils, egg yolk, dairy products, spinach, pumpkins, carrots, mangoes, papayas, grains, and other fruits and vegetables (FAO/WHO, 2004). The requirements have been set for adults, adolescents, children and infants. However, no requirements have been set for antioxidants obtained from fruits, vegetables, and beverages such as tea, as this would pose an almost impossible task. Vitamins A, C and E are crucial in preventing and combating childhood infectious diseases such as diarrhoea, respiratory infections and measles. Adults, on the other hand, may be burdened by non-communicable chronic diseases such as cardiovascular disease, diabetes and cancer, which are a growing concern in low income countries (Anderson & Chu, 2007). A common characteristic of these diseases is the presence of oxidative stress that causes membrane damage, inflammation, and cellular dysfunction. As a result of low intake of fruits and vegetables in South Africa (Vorster *et al*., 1997), and that vitamin A, C and E intakes are insufficient to meet the requirements (Labadarios *et al*., 2005), it can be assumed that antioxidant status of South Africans are low. This increases their risk for childhood infections and non-communicable chronic disease. It should be noted that these diseases are multi-faceted diseases, and that only the role of antioxidants are explored in this study.

Tea does not provide vitamins A, C or E, however is abundant in polyphenolic compounds which are antioxidants that offer protection by defending against free-radical damage (Pietta,

2000). Traditional black and Rooibos tea is used by different communities and is introduced into the diet of infants as young as 6 months. Tea cannot necessarily be used as a treatment for childhood infections or non-communicable chronic disease; however it can be used as part of a prevention plan to increase the antioxidant status. Tea is therefore an affordable, easily obtainable and long shelf-life product that can be used to increase antioxidant intake of South Africans.

The eventual antioxidant activity of tea radical scavengers is dependent on the digestive environment that results in biotransformation of flavanols and other flavonoids in the stomach, small intestine and colon of the GIT (Spencer, 2003). The antioxidant activity of different types of tea has been extensively evaluated and the bio-active molecules have been identified. After consumption, tea is subjected to GIT digestion which includes stomach and duodenal digestion. The GIT can have variable effects on the antioxidant properties of tea. This includes increased extraction of polyphenolic compounds in an acidic environment, causes the selective inactivation or degradation of specific flavonoids, or may even have no effect. Alternatively the polyphenolics can bind protein which can have variable effects on activity. Polyphenols may interact with globular proteins and form non-covalent bonds that result in complexation (Chaudhuri *et al*., 2011), protein structure stabilization (Kanakis *et al*., 2011), protein unfolding and precipitation. The size and structure of polyphenols and amino-acid sequence of proteins determines the strength of interactions (Frazier *et al*., 2010). Antioxidant properties of polyphenols can be influenced when polyphenols form complexes with proteins, due to the polyphenols' electron donating ability and the number of hydroxyl groups available in solution being reduced (Arts *et al*., 2001).

Consumption of tea in nutritional based studies have shown that polyphenols are present in urine, faeces and blood plasma, however the quantities present in the aforementioned are very small in comparison to the amounts ingested (Rietveld & Wiseman, 2003). From these studies it is assumed that digestion may have some effect on activity, but a significant portion of antioxidant molecules are still bio-available and once in the blood can protect by scavenging free radicals and chelating metals that are responsible for lipid peroxidation (Bravo, 1998). A portion of these antioxidant molecules such as the theaflavins (Yang, Chen & Wu, 2014) are practically not bioavailable but still can protect against chronic inflammation associated GIT disorders such as

gastroesophageal reflux disease and inflammatory bowel disease. Inflammation is associated with chronic oxidative stress, which can lead to the development of cancer within the GIT (Qiao & Li, 2014). Antioxidants can reduce oxidative stress thereby reducing the risk for these diseases.

To the author's knowledge no studies have been done that compare adult and infant digestion of either black tea or Rooibos tea, either *in vivo* or *in vitro*. Adult digestion of Rooibos tea and infant digestion of black and Rooibos tea has also not been studied in terms of its effect on antioxidant activity. This study was undertaken to investigate, in the South African context, the antioxidant activity before and during digestion of *Camellia sinensis* and *Aspalathus linearis* tea. As these teas are also consumed by infants, the effects of infant and adult GIT conditions on activity were also investigated.

6.2 Summary of main findings

Four commercial brands of black tea and 3 of Rooibos tea were selected and water extracts based on traditional South African methods of tea preparation were used. The TPC and TFC of fermented *Camellia sinensis* was 3.11 and 1.53 times greater than fermented *Aspalathus linearis* tea. Antioxidant activity measured with the DPPH, TEAC and ORAC assays showed that the antioxidant activity of fermented *Camellia sinensis* tea was 2.74, 2.95 and 2.06 times greater than fermented *Aspalathus linearis* tea, respectively. Both teas effectively scavenged NO and this effect was 1.96 times better for fermented *Camellia sinensis*. Caco-2 cells were protected against AAPH-induced oxidative damage by fermented *Camellia sinensis* and *Aspalathus linearis* tea and this effect was 1.14 fold greater for *Camellia sinensis* tea. Based on the calculations of du Toit *et al*. (2001) in this study approximately one cup of black tea and two cups of Rooibos tea would be sufficient to meet 200 mg vitamin C equivalents, antioxidant intake.

Hypothesis 1 is accepted which states that the antioxidant content and antioxidant activity, NO scavenging activity and cellular antioxidant activity of locally consumed fermented *Camellia sinensis* and *Aspalathus linearis* teas does contribute significantly to the antioxidant status of the South African diet.

With adult digestion, the low pH of SD causes an increase in the extraction of fermented *Camellia sinensis* polyphenolics which with further digestion decreases by 28.57% compared to UD. No changes in TFC levels were found with adult digestion. Antioxidant activity of adult digested black tea measured with the DPPH assay decreased by 31.19% for SD and for SDD 62.50% compared to UD. Activity that remained following digestion was 52.38%. With the TEAC assay the measured antioxidant activity decreased by 24% for SD and for SDD was unchanged compared to UD and the activity that was retained was 94.64% of UD. Further evaluation of antioxidant activity with the ORAC assay showed that with SD and SDD activity decreased by 13.32% and 37.77% compared to UD respectively. Remaining activity following SDD was 68.23%. NO scavenging activity of fermented *Camellia sinensis* tea was unchanged with SD but was decreased by 20.58% with SDD compared to UD. Cellular antioxidant activity, compared to UD, was decreased by 2.96% with SD and 15.09% with SDD. Remaining activity was 83.15%.

For adult digestion of fermented *Aspalathus linearis* tea, TPC increased with a non-significant 10.53% following SD but with SDD decreased to it's original UD value, with 100% content remaining. In contrast SD and SDD resulted in a 20.56% and a 10.71% decrease in TFC compared to its UD content. Antioxidant activity of digested Rooibos tea measured with the DPPH assay decreased by 59.15% for SD and 38.96% for SDD compared to UD and 67.39% activity remained following SDD. SD and SDD antioxidant activity decreased by 45.16% and 11.11% respectively, compared to UD, when measured with the TEAC assay. Remaining activity after digestion was 89.47%. The ORAC assay revealed that antioxidant activity was reduced by 12.09% and 41.50% for SD and SDD respectively, compared to UD remaining activity was 65.63%. NO scavenging activity of Rooibos tea decreased by 15.47% and 41.55% for SD and SDD respectively, compared to UD. Cellular antioxidant activity was decreased by 0.32% and 16.55% for SD and SDD respectively with the remaining activity being 71.71%. In general the antioxidant content and activity, NO scavenging activity as well as the cellular antioxidant activity of *Camellia sinensis* tea were greater than *Aspalathus linearis* tea. However both retain antioxidant activity, NO scavenging ability which results in significant cellular protection against oxidants.

Interestingly, the presence of proteins such as the digestive enzymes seems to modulate measured antioxidant activity either by possibly quenching free radicals or by directly binding polyphenols.

Hypothesis 2: Using an *in vitro* simulated adult digestion model, the measured antioxidant, NO scavenging and cellular protection parameters for fermented *Camellia sinensis* and *Aspalathus linearis* teas remain stable with SD and are reduced with SDD and these differences are mainly due to the effect of pH was only partially proven. Variable results for TPC, TFC and antioxidant activity were measured with the DPPH, TEAC and ORAC assays and these differences are due to the types, structure and activity of the polyphenolics found in *Camellia sinensis* and *Aspalathus linearis* teas. In addition to pH, the gastric enzymes also affect activity possibly by either binding the polyphenolics or quenching ROS.

The effect of infant digestion was then evalauted. For infant digested fermented *Camellia sinensis* tea, the low pH of SD resulted in a 16.39% increase in the extraction of polyphenolics which then decreased with SDD to 33.33% less than the UD value. Throughout digestion, TFC was unchanged. Compared to UD, antioxidant activity measured with the DPPH assay was unaltered for SD and for SDD reduced by 32.26% compared to UD and remaining activity following SDD was 72.22%. With the TEAC assay the measured antioxidant activity decreased by 13.33% for SD and for SDD was unchanged compared to UD. Following SDD the remaining antioxidant activity was 101.79% of UD. With the ORAC assay antioxidant activity compared to UD was reduced by 24.67% and 53.69% for SD and SDD respectively. The remaining activity was 57.67%. NO scavenging activity of black tea was reduced by 12.89% and 11.33% for SD and SDD respectively. Evaluation of cellular antioxidant activity revealed that activity was unchanged for SD and decreased by 1.83% following SDD and remaining activity was 94.97%.

For infant digested fermented *Aspalathus linearis* tea, TPC was increased and with SDD decreased by 25.00% compared to UD tea. Remaining TPC was 77.78%. With digestion TFC levels were reduced slightly to 94.92% of UD tea. Antioxidant activity, measured with the DPPH assay showed an increase of 14.14% for SD and which decreased by 21.69% compared to UD following SDD. Remaining activity following digestion was 80.43%. With the TEAC assay with SD and SDD antioxidant activity decreased by 17.14% and 5.41% respectively compared to UD and remaining activity was 94.74%. Antioxidant activity measured with the ORAC assay

revealed that antioxidant activity was reduced by 35.61% and 45.34% for SD and SDD respectively. The remaining activity compared to UD was 63.04%. NO scavenging activity was decreased by 25.70% and 16.33% for SD and SDD respectively, compared to UD tea. Cellular antioxidant activity was decreased by 2.45% for SD and was unchanged for SDD with 83.99% activity being retained. As for adult digestion, the presence of proteins such as the digestive enzymes seems to modulate measured antioxidant activity either by possibly quenching free radicals or by directly binding polyphenols.

Hypothesis 3: As for adult digestion black tea consistently showed better activity than Rooibos tea; however both retained a considerable amount of antioxidant activity, NO scavenging ability which resulted in cellular protection against oxidants. As for hypothesis 2, hypothesis 3 that states that with an *in vitro* simulated infant digestion model, the measured antioxidant, NO scavenging and cellular antioxidant activity for *Camellia sinensis* and *Aspalathus linearis* teas remain stable with SD and are reduced with SDD and these differences are also due to the effect of pH was only partially proven. pH did have an effect on measured parameters although to a lesser degree than adult digestion. In addition to pH, the gastric enzymes also contributed to measured activity by either binding the polyphenolics or quenching ROS.

6.3 Implications of the study

Antioxidant content and activity, NO scavenging ability as well as cellular antioxidant activity of *Camellia sinensis* and *Aspalathus linearis* tea was altered with digestion, however following SDD, significant levels of antioxidant properties were retained. Nutritionally the amount of activity is significant and can impact on the oxidative status of cells and tissues.

A poor antioxidant status is associated with high levels of ROS that is associated with the development of cancer, cardiovascular and inflammatory diseases, and aging (Dufresne & Farnworth, 2001). Polyphenolic compounds, particularly flavonoids, defend against oxidation (Dufresne & Farnworth, 2001). Tea is rich in polyphenols, which have antioxidant, anti-cancer, antimicrobial and anti-inflammation properties and therefore tea is a beneficial product that can prevent the development of cancer, cardiovascular and inflammatory diseases (Dufresne & Farnworth, 2001; Laparra & Sanz, 2010).

Although the bio-availability of polyphenolics from the gut is poor, polyphenolics in the GIT can protect the mucosa against oxidative damage. Some tea phenolics are transported into the colon, where these molecules interact with microflora of the gut (Laparra & Sanz, 2010). Gut microflora metabolise polyphenolic compounds, breaking the glycoside bonds and changing them to aglycones, which are then better absorbed in the intestine. Many of the unabsorbed metabolites display anti-inflammatory, antimicrobial or bacteriostatic properties and can selectively inhibit growth of pathogens and increase the growth of commensal bacteria (Laparra & Sanz, 2010). In addition these polyphenolics can reduce inflammation in the GIT and reduced the risk of GIT associated cancer.

In this study the measured antioxidant parameters were the best for black tea when compared to Rooibos tea. However, black tea contains caffeine whereas Rooibos tea is naturally caffeine-free. In the body, several metabolizing systems exist for the degradation and elimination of caffeine. These include cytochrome P-450 (CYP) enzymes, phase II conjugation systems, serum esterases and epoxide hydrolase. In adults these enzyme systems are fully developed while at birth these metabolizing systems are immature and develop at varying rates during infancy (Ginsberg *et al*., 2004). This results in slower clearance of caffeine from infants and children's system. The main pathway of clearance of caffeine by means of N^3-demethylation is CYP1A2 (Ginsberg *et al*., 2004). In addition to this, genetic variation amongst individuals also plays a role in caffeine metabolism. Individuals who are homozygous for the CYP1A2*1A allele metabolise caffeine rapidly, while those who have the variant CYP1A2*1F metabolise caffeine slowly (Cornelis *et al*., 2006). Studies have found that more than 50% of Caucasians in Africa have a genetic polymorphism for CYP1A2 (Aklillu *et al*., 2007). Caffeine may cause sleeplessness, agitation, anxiety, gastrointestinal disturbances and arrhythmias. Infants and children need adequate sleep for optimal growth and development (Seifert *et al*., 2011). Therefore, even though fermented *Camellia sinensis* shows better antioxidant properties with adult and infant digestion, based on the effects of caffeine Rooibos tea should be given to infants and children and adults sensitive to the effects of caffeine. In children black tea should be given infrequently and long before expected nap times.

6.4 Limitations

Even though tea bags are widely used in South Africa because of convenience, loose tea leaves are also used. Tea leaves may contain higher levels of antioxidant content and activity than tea bags, and may have exhibited a greater difference regarding the effects of digestion. The brands of tea used in this study are representative of the tea types consumed in South Africa. Different batches of the tea samples were not used, and therefore does not account for variation in production, season and blends, and consequently antioxidant activity. However these differences may not have a major impact on the measured activity.

More control samples could have been used, however as this was a South African based study, this may not be necessary. Green tea and unfermented Rooibos tea are not widely consumed and therefore were used only for comparative purposes.

A simulated *in vitro* model was used in our study, as opposed to *in vivo* digestion. Although simplistic, an *in vitro* model does provide information on the probable effects of digestion. Simulated *in vitro* methods are meant to provide a rapid testing method for food ingredients, however is not going to achieve the accuracy of an *in vivo* system due to the inherent complexity of the *in vivo* process (Hur *et al*., 2011). *In vivo* digestion encompasses a complex system that involves bile, lipase, amylases, gastrointestinal transit times, and mechanical digestion/peristalsis (Hur *et al*., 2011). Food matrix interactions, such as those between milk protein and tea polyphenolic compounds, or even medication, may alter digestion of tea *in vivo* (McClements *et al*., 2008). All these factors play a role in the breakdown, degradation and perhaps even the antioxidant activity of tea polyphenols. The disadvantage of using an animal model is that it is time consuming and costly (Hur *et al*., 2011). Animals also have different energy and food intakes, lifespan and body proportion, and physiological differences to humans (García & Díaz-Castro, 2013). Research findings done on animals can therefore not be directly extrapolated to humans. Further testing using *in vitro* cell models will be of value, and cellular protective effects can also be determined using different cell lines. Once these types of studies are completed an appropriate animal model can be identified for further experimentation.

The ability of the bio-active components in tea to reduce NO production in a cellular model can be further investigated. A typical model used for this type of assessment of activity is the model used by Kim *et al*. (1999), which will then measure the ability of tea extracts to reduce LPS

stimulated NO production using the RAW 264.7, murine monocyte/macrophage cell line. The effect of the tea polyphenolics on inducible nitric oxide synthase (iNOS) activity can then be determined.

6.5 Recommendations

Future studies should include more samples, of teabags and tea leaves, fermented and unfermented samples, in order to have a more representative sample and to compare the differences in antioxidant properties of teabags and tea leaves.

Cell models could be used to determine bioavailability of phenolic compounds. For this purpose, a Caco-2 monolayer may be used to determine bioavailability of tea samples after digestion (Artursson, Palm & Luthman, 2001). Cell lines are cultivated as monolayers on permeable filters in order to evaluate transepithelial transport of compounds, which can model the transport of phenolic compounds *in vivo* (Artursson *et al*., 2001). The bio-availability of polyphenolics following each phase of digestion can then be evaluated in this model. It is important to determine the effect of digestion including the effect of pH and the presence of protein i.e. on the concentration and levels of polyphenolics found in SD and SDD using HPLC-MS. Milk and sugar/honey are often added to tea, matrix interactions should be further investigated between the milk protein, casein, carbohydrate compounds and polyphenolic compounds present in tea. The effect on the availability and antioxidant activity can also be analysed in these mixed samples.

It is recommended that further investigation be carried out to determine the effect of adult and infant digestion of fermented Rooibos tea and black tea on individual phenolic compounds present in tea. This may explain further the increases and decreases observed during stomach and duodenal digestion in this research. Further investigation should also be carried out to determine what effect the pH change of infant (pH 4 to pH 7) and adult (pH 2 to pH 7) digestion has on the individual phenolic compounds in terms of quantity and their structure. HPLC-MS can then be used on the undigested, digested and pH control samples to analyse the phenolic composition of the samples and the changes caused therein by digestion of black and Rooibos tea. HPLC-MS analysis will allow the identification of each phenolic compound, and the effect of pH and pH with digestive enzymes on molecular structure and stability can be determined.

6.6 Conclusions

In this laboratory based investigation fermented *Camellia sinensis* and *Aspalathus linearis* was shown to be rich in polyphenolics with high antioxidant activity, NO scavenging activity and cellular antioxidant properties. With digestion measured parameters are increased, unchanged or reduced. In instances where the antioxidant properties of the gastric fractions are reduced the remaining activity is enough to ensure significant levels of protection against ROS, implying that both fermented Rooibos and black tea may assist in the prevention and management of oxidative damage associated with infections and chronic diseases.. Although pH affects activity the presence of gastric enzymes also alters activity. Although fermented *Camellia sinensis* had better antioxidant properties than *Aspalathus linearis* tea, the presence of caffeine for infants, children and susceptible adults is a better option.

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Addendum

A COMPARISON OF TOTAL ANTIOXIDANT CONTENT AND ACTIVITY OF 4 COMMERCIALLY AVAILABLE SOUTH AFRICAN TEAS AND THEIR PROTECTION AGAINST OXIDATIVE DAMAGE

P Müller, A Oelofse, MJ Bester and KG Duodu. Centre for Nutrition, Department of Food Science, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa.

Oxidative stress due to reactive oxygen species (ROS) and free radicals cause extensive damage to DNA, proteins and lipids, contributing to aging and degenerative diseases such as cancer, cardiovascular disease and diabetes. Tea is abundant in polyphenolic compounds, particularly flavonoids, which are antioxidants that offer protection by various mechanisms that involve suppressing ROS, scavenging ROS, and by protecting antioxidant defences. The antioxidant content and activity and of 4 commercially available teas were determined, as well as their ability to protect against oxidative damage in a cellular environment. Total polyphenolic (TPC) and flavonoid (TFC) content was determined by Folin-Ciocalteu and aluminium chloride assays, respectively, whereas antioxidant activity was determined by DPPH radical scavenging, TEAC and ORAC assays. Oxidative damage was determined by Caco-2 cell models. Black tea had the highest antioxidant content (105.35 mg GAE/g; 89.87 mg CE/g), followed by green tea (83.33 mg GAE/g; 71.67 mg CE/g), unfermented Rooibos tea (51.70 mg GAE/g; 68.56 mg CE/g), and fermented Rooibos tea (38.89 mg GAE/g; 59.30 mg CE/g). The same trend was observed in the TEAC and ORAC assays, whereas in the DPPH assay green tea had the highest antioxidant activity (129.88 mg TE/g), followed by black tea (125.68 mg TE/g), unfermented Rooibos tea (64.30 mg TE/g), and fermented Rooibos tea (46.20 mg TE/g). Teas did not differ significantly in protection against oxidative damage on a cellular level, offering 80% or more protection against oxidative damage, proving the effectiveness of tea as antioxidants that should be consumed as part of a healthy balanced diet.

EFFECT OF DIGESTION OF *CAMELLIA SINENSIS* AND *ASPALATHUS LINEARIS* TEAS ON ANTIOXIDANT CONTENT AND ACTIVITY AND THEIR PROTECTION AGAINST OXIDATIVE DAMAGE

P Müller, A Oelofse, MJ Bester and KG Duodu. Centre for Nutrition, Department of Food Science, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa.

Low antioxidant status of people due to not consuming adequate fruits and vegetable is currently a major concern. Despite this, tea remains the main source of antioxidants, particularly flavonoids. The ultimate antioxidant potential of these radical scavengers is dependent on digestion conditions that results in biotransformation of flavanols and other flavonoids in the stomach, small intestine and colon of the gastrointestinal tract. The effect that digestion of tea has on antioxidant content and activity, as well as protective ability against oxidative damage in a cellular environment was determined. The Folin-Ciocalteu and aluminium chloride assays were done to determine total polyphenolic (TPC) and flavonoid (TFC) content, respectively, whereas DPPH radical scavenging, TEAC and ORAC assays were used to determine antioxidant activity. Caco-2 cell models were used to measure oxidative damage. *Camellia sinensis* teas had higher antioxidant contents and activities than *Aspalathus linearis* teas, however on a cellular level they did not differ significantly in protection against oxidative damage. Pepsin (stomach) and pancreatin (intestines) digestion drastically reduced the TPC and radical scavenging ability, however TFC was minimally affected. Total antioxidant activity (TEAC) decreased with pepsin digestion and increased with further pancreatin digestion. After pepsin and pancreatin digestion, teas still had powerful protective effects against oxidative damage on a cellular level. Neutral and alkaline pH environments of the small intestine and colon cause polyphenolic compounds to oxidise. Oxidised products sometimes possess greater radical scavenging activity than their original flavonoids. Thus the value of tea as a potent antioxidant source should not be underestimated.